Published in final edited form as:

Pain. 2010 December ; 151(3): 843–852. doi:10.1016/j.pain.2010.09.026.

Fracture induces keratinocyte activation, proliferation, and expression of pronociceptive inflammatory mediators

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Abstract

Tibia fracture in rats results in chronic vascular and nociceptive changes in the injured limb resembling complex regional pain syndrome (CRPS) and up-regulates expression of interleukin 1 β (IL-1 β), interleukin IL-6 (IL-6), tumor necrosis factor- α (TNF- α), and nerve growth factor- β (NGF- β) in the hindpaw skin. When fracture rats are treated with cytokine or NGF inhibitors nociceptive sensitization is blocked. Because there is no leukocyte infiltration in the hindpaw skin we postulated that resident skin cells produce the inflammatory mediators causing nociceptive sensitization after fracture. To test this hypothesis rats underwent distal tibia fracture and hindlimb casting for 4 weeks, then the hindpaw skin was harvested and immunostained for keratin, cytokines and NGF. BrdU staining was used to evaluate cell proliferation. Hindpaw nociceptive thresholds, edema, and temperature were tested before and up to 96 hours after intraplantar injections of IL-6 and TNF- β . Tibia fracture caused keratinocyte activation, proliferation, and up-regulated IL-1, IL-6, TNF- α and NGF- β protein expression in the hindpaw keratinocytes. Local injections of IL-6 and TNF- α induced hindpaw mechanical allodynia lasting for several days and modest increases in temperature and edema. These data indicate that activated keratinocytes proliferate and express IL-1 β , IL-6, TNF- α , and NGF- β after fracture and that excess amounts of inflammatory mediators in the skin cause sustained nociceptive sensitization. This is the first study demonstrating in vivo keratinocyte expression of IL-6, TNF- α and NGF- β in a CRPS model and we postulate that the keratinocyte is the primary cellular source for the inflammatory signals mediating cutaneous nociceptive sensitization in early CRPS.

Keywords

Complex regional pain syndrome; keratinocytes; inflammation; cytokine; nerve growth factor; Substance P

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1. Introduction

Complex regional pain syndrome (CRPS) is a painful, disabling and often chronic condition affecting the extremities and is a frequent sequelae of distal tibia [45] and radius fractures [4]. Recently we described a distal tibia fracture model in rats that exhibits chronic unilateral hindlimb warmth, edema, facilitated spontaneous protein extravasation, allodynia, unweighting, and periarticular osteoporosis [18]. This constellation of post-fracture changes closely resembles the clinical presentation of patients with acute CRPS.

The inflamed appearance of the affected CRPS limb has led to the hypothesis that the local production of inflammatory mediators might be involved in the etiology of the condition. In support of this idea, treating fracture rats with the global cytokine inhibitor pentoxifylline, a TNF inhibitor (etanercept), an IL-1 receptor antagonist (anakinra) or an anti-NGF antibody (tanezumab) blocked the development of hindpaw allodynia and unweighting at 4 weeks post-fracture [31;42;43;55]. All these drugs (except pentoxifylline) are large molecular weight proteins that cannot cross the blood brain barrier, suggesting a peripheral site of action. These treatments also inhibited the increase in spinal cord Fos expression characterizing this and other rodent models of painful conditions [42;43]. In addition, at 4 weeks after fracture there was a dramatic increase in hindpaw skin expression IL-1 β , IL-6, TNF- α , and NGF- β , measured at both the mRNA and protein levels [42;43;55]. IL-6 and TNF- α levels are elevated in suction blister fluid obtained from the skin of the affected limb, when compared to the contralateral side, in CRPS patients [21;22]. Although cutaneous inflammatory mediator expression appears up-regulated in the CRPS limb, there is no evidence of classical immune system activation in these patients [9;26;41]. Similarly, no leukocyte infiltration is observed in the hindpaw skin at 4 weeks post-injury in the CRPS fracture model [55]. Based on these data we suspected that resident cells in the skin might be the primary source for the inflammatory mediators observed after fracture and in CRPS patient skin.

Dysregulation and abnormal expression of inflammatory mediators or their receptors in keratinocytes is relevant to the pathogenesis of several chronic inflammatory skin diseases such as psoriasis, atopic dermatitis and allergic contact dermatitis [1;2;13;35]. Thus we hypothesized that keratinocyte activation and proliferation in the CRPS fracture model could lead to the enhanced expression of inflammatory mediators in the skin, leading to nociceptive sensitization, warmth, and edema. We previously evaluated the nociceptive and vascular effects of IL-1 β and NGF intraplantar injections in naïve rats and observed a dose-dependent long-lasting hyperalgesic effect for both inflammatory mediators [31], but we had not tested the effects of IL-6 and TNF- α intraplantar injections in naïve rats. The objectives of the current study were to determine the cellular source for the cutaneous inflammatory mediators expressed in the fracture hindlimb and to test the nociceptive and vascular effects of local IL-6 and TNF- α injection. We show that tibia fracture causes local activation of skin keratinocytes leading to expression of pronociceptive inflammatory mediators that could potentially contribute to complex regional pain syndrome.

2. Materials and methods

These experiments were approved by our Institutional Animal Care and Use Committee and followed the animal subjects' guidelines of the IASP[60]. Adult (9-month-old) male Sprague Dawley rats (Simonsen Laboratories, Gilroy, CA) were used in all experiments. The animals were housed individually in isolator cages with solid floors covered with 3 cm of soft bedding and were given food and water ad libitum. During the experimental period the animals were fed Lab Diet 5012 (PMI Nutrition Institute), which contains 1.0% calcium, 0.5% phosphorus, and 3.3 IU/g of vitamin D₃, and were kept under standard conditions with a 12-h light-dark cycle.

2.1 Surgery

Tibia fracture was performed under isoflurane anesthesia as we have previously described [18]. The right hindlimb was wrapped in stockinet (2.5 cm wide) and the distal tibia was fractured using pliers with an adjustable stop (Visegrip, Petersen Manufacturing) that had been modified with a 3-point jaw. The hindlimb was then wrapped in casting tape (Delta-Lite, Johnson & Johnson) so the hip, knee and ankle were flexed. The cast extended from the metatarsals of the hindpaw up to a spica formed around the abdomen. The cast over the paw was applied only to the plantar surface; a window was left open over the dorsum of the paw and ankle to prevent constriction when post-fracture edema developed. To prevent the animals from chewing at their casts, the cast material was wrapped in galvanized wire mesh. The rats were given subcutaneous saline and buprenorphine immediately after procedure (0.03 mg/kg) and on the next day after fracture for post-operative hydration and analgesia. At 4 weeks the rats were anesthetized with isoflurane and the cast removed with a vibrating cast saw. All rats used in this study had union at the fracture site after 4 weeks of casting.

2.2 Drug treatments

Nociceptive and vascular responses to intraplantar injection of IL-6, and TNF- α (Sigma) were assessed in control rats by subcutaneous injection using a 27 gauge needle and microsyringe (Hamilton, Las Vegas, NV) and gentle restraint. The cytokines were diluted in sterile phosphate buffered saline (PBS) and injected the doses below chosen on the basis of preliminary dose-response studies demonstrating hyperalgesic effects: IL-6: 1ng/50 μ l, 10ng/50 μ l, and 60ng/50 μ l; TNF- α : 1ng/50 μ l, 10ng/50 μ l, and 70ng/50 μ l.

2.3 In vivo BrdU labeling and BrdU immunohistochemistry

Labeling with BrdU was done to evaluate keratinocyte proliferation. At 3 weeks after tibia fracture, animals were injected intraperitoneally once daily with 50 mg/kg BrdU (Sigma-Aldrich) for 8 days [56]. Hindpaw skin was harvested and fixed one day after the last injection and processed for immunostaining [56]. Skin sections were pretreated in 2 N HCl for 30 min at 37 °C, followed by neutralization in 0.1 M borate buffer (pH 8.5) for 10 min and blocking with 10% normal donkey serum for 1 hr at room temperature, after which immunohistochemistry was performed using a rat anti-BrdU monoclonal antibody (1:300, Accurate Chemical) and donkey anti-rat fluorescein isothiocyanate secondary antibody (1:300, Jackson ImmunoResearch Laboratories). BrdU immunostaining was observed using a Leica DM 2000 fluorescent microscope and imaged using Spot Camera (version 4.0.8, Diagnostic Instruments). The number of BrdU-positive cells were counted specifically in the keratin positive cells in the area of the epidermis, with a minimum of six sections per animal from seven intact and five fracture animals. Cell densities were calculated by dividing cell numbers by the area. Representative images were obtained using confocal microscopy (Zeiss LSM/510 Upright 2 photon; Carl Zeiss).

2.4. Hindpaw nociception

To measure mechanical allodynia in the rats an up-down von Frey testing paradigm was used as we have previously described [18;25]. Rats were placed in a clear plastic cylinder (20 cm in diameter) with a wire mesh bottom and allowed to acclimate for 15 min. The paw was tested von Frey fibers ranging in stiffness from 0.41 to 15.14 g. The von Frey fiber was applied against the hindpaw plantar skin at approximately midsole, taking care to avoid the tori pads. The fiber was pushed until it slightly bowed and then it was jiggled in that position for 6s. Stimuli were presented at an interval of several seconds. Hindpaw withdrawal from the fiber was considered a positive response. Withdrawal thresholds were calculated according to the method of Poree [39].

An incapacitance device (IITC Inc. Life Science) was used to measure hindpaw unweighting. The rats were manually held in a vertical position over the apparatus with the hindpaws resting on separate metal scale plates. This allowed measurement of the weight resting on each hind paw independently. The duration of each measurement was 6 s, and 10 consecutive measurements were taken at 60-s intervals. Eight readings (excluding the highest and lowest ones) were averaged to calculate the bilateral hindpaw weight bearing values.

2.5. Hindpaw thickness

A laser sensor technique was used to determine the dorsal-ventral thickness of the hindpaw over the midpoint of the third metatarsal as we have previously described [18;19;25]. For laser measurements each rat was briefly anesthetized with isoflurane and then held vertically so the hindpaw rested on a table top below the laser. The paw was gently held flat on the table with a small metal rod applied to the top of the ankle joint. Using optical triangulation, a laser with a distance measuring sensor was used to determine the distance to the table top and to the top of the hindpaw and the difference was used to calculate the dorsal-ventral paw thickness. The measurement sensor device used in these experiments (4381 Precicura, Limab) has a measurement range of 200 mm with 0.01 mm resolution.

2.6. Hindpaw temperature

The temperature of the hindpaw was measured using a fine wire thermocouple (Omega) applied to the paw skin, as previously described [18;19;25]. The investigator held the thermistor wire using an insulating Styrofoam block in the jaws of fine forceps. Three sites were tested over the dorsum of the hindpaw; the space between the first and second metatarsals (medial), the second and third metatarsals (central), and the fourth and fifth metatarsals (lateral). After a site was tested in one hindpaw the same site was immediately tested in the contralateral hindpaw. The testing protocol was medial dorsum right then left, central dorsum right then left, lateral dorsum right then left, medial dorsum left then right, central dorsum left then right, and lateral dorsum left then right. The six measurements for each hindpaw were averaged for the mean temperature.

2.7. Tissue processing and immunofluorescence confocal microscopy

Animals were euthanized and perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4, via the ascending aorta; the hindpaw skin including sub-dermal layers was removed and post-fixed in 4% PFA for 2 hours, then the tissues were treated with 30% sucrose in PBS at 4°C before embedding in OCT (Sakura Finetek). Following embedding, 20- μ m thick slices were made using a cryostat, mounted onto Superfrost microscope slides (Fisher Scientific), and stored at -70°C.

Frozen sections were permeabilized and blocked with PBS containing 10% donkey serum and 0.3% Triton X-100, followed by exposure to the primary antibodies overnight at 4°C in PBS containing 2% serum. Upon detection of the first antigen, primary antibody from a different species against the second antigen was applied to the sections and visualized using an alternative fluorophore-conjugated secondary antibody. Sections were then rinsed in PBS and incubated with fluorophore-conjugated secondary antibodies against the immunoglobulin of the species from which the primary antibody was generated. After three washes, the sections were mounted with anti-fade mounting medium (Invitrogen). Images were obtained using confocal microscopy (Zeiss LSM/510 Upright 2 photon; Carl Zeiss) and stored on digital media. The sources of primary antibodies were as follows: goat anti-rat IL-1 β , 1:200 (R&D Systems), goat anti-rat IL-6, 1:100 (R&D Systems), goat anti-rat NGF- β , 1:100 (R&D Systems) and monoclonal mouse anti-rat keratin, Pan Ab-1, 1:50 (clone AE1/AE3) (Thermo Fisher Scientific). The sources of secondary antibodies were as follows: donkey anti-mouse IgG (1:300) conjugated with cyanine dye 3, or donkey anti-goat IgG (1:500) conjugated with

fluorescein (FITC) secondary antibodies (Jackson ImmunoResearch Laboratories), incubated with respective primary antibodies. Control experiments included incubation of slices in primary and secondary antibody-free solutions and primary antibody pre-absorption control, all of which led to low intensity non-specific staining patterns in preliminary experiments (data not shown). For pre-absorption controls, 250–500 ng of the primary antibody incubated with 1–2 µg of the corresponding immunizing antigen in 500 µL of PBS at 4°C overnight, before it was used for labeling.

Immunostaining for TNF- α was approached differently. TSA Plus Fluorescence Systems (Perkin Elmer LAS) were used for TNF- α immunostaining. Briefly, incubation and washing procedures were carried out at room temperature if they were not specified. The glass slides were washed in PBS containing 0.2% Triton X-100 (two times, 15 min each) and incubated with 300 µL of TNB buffer (0.1 mol/L Tris-HCl, pH 7.5; containing 0.15 mol/L NaCl and 0.05% Tween 20 and 0.5% NEN blocking reagent) for 1 hr followed by incubation with rabbit anti-TNF alpha polyclonal antibody (Diluted in TNB buffer, 1:100, ABR-Affinity BioReagents) overnight at 4°C. The glass slides were then washed in TNT buffer three times, 5 min each and the slides were then incubated with 300 µL of HRP labeled secondary antibody (diluted in TNB buffer 1:300) for 30 min, followed by washing in TNT (three times, 5 min each). Subsequently, 300 µL of Fluorophore Tyramide (fluorescein amplification reagents, 1:50 diluted in 1× plus amplification diluent) was applied for 3 to 5 minutes. After washing in TNT (six times, 5 min each), slides were incubated with 10% donkey serum for 1 hr followed by incubated with mouse anti-rat keratin, Pan Ab-1, 1:50 (clone AE1/AE3) (Thermo Fisher Scientific) containing 2% serum overnight at 4°C. The slides were then rinsed in PBS followed by incubated with donkey anti mouse IgG (1:400) conjugated with cyanine 3 secondary antibody (Jackson ImmunoResearch Laboratories). After rinsing in PBS (three times, 5 min each), the sections were mounted with anti-fade mounting medium (Invitrogen). Images were obtained using confocal microscopy (Zeiss LSM/510 Upright 2 photon; Carl Zeiss) and stored on digital media. Quantitative studies were based on four or more replicates. Each inflammatory mediator-positive keratinocytes were counted per high-power field (HPF, 400×) in the epidermis in hindpaw skins of 5 fracture and 5 control rats. Student's *t*-test was used to determine the *P* value for statistical significance.

2.8 Statistical analysis

Behavioral data collected over time after intraplantar injections of each inflammatory mediator were analyzed by repeated measures ANOVA on data for each test time point, comparing various treatment groups, where the repeated measure was time post-injection. A Bonferroni test was used to determine the source of differences between each dose for each inflammatory mediator. For simple comparisons of two means, two-tailed *t*-testing was performed. All data are presented as the mean $\pm$ SE of the mean, and differences are considered significant at a *p* value less than 0.05 (Prism 4, GraphPad Software).

3. Results

3.1. Fracture induced hindpaw keratinocyte proliferation

We previously observed that distal tibia fracture is accompanied by an increase in hindpaw skin thickness [54]. Additionally, trophic changes including skin thickening is common in CRPS [20]. Therefore, we hypothesized that hindpaw keratinocyte proliferation would be enhanced by fracture. To test this hypothesis, BrdU was used to label dividing keratinocytes. Fig. 1A shows representative confocal images of BrdU (green) and keratin (red) in hindpaw skin sections from sham operated control rats and from the hindpaws ipsilateral to fracture 4 weeks post-injury. Although a few BrdU positive cells were detected in control skin (Fig. 1A upper panel), skin sections from fracture rats demonstrated a larger proportion of positive cells

in the epidermis which co-labeled for keratin including in the basal layer where new keratinocytes are generated (Fig. 1A lower panel). Quantification of the BrdU-positive cells in hindpaw skin revealed a 350% increase in BrdU positive cells in the fracture limb (6.2 ± 0.4 cells/ $10^3 \mu\text{m}^2$, $n=5$), but no change was observed in the contralateral paw (2.2 ± 0.1 cells/ $10^3 \mu\text{m}^2$, $n=5$), as compared to skin from sham operated controls (1.8 ± 0.2 cells/ $10^3 \mu\text{m}^2$, $n=7$) (Fig. 1B). Consistent with these results, Fig. 1C illustrates a 270% increase in the thickness of the epithelial keratinocyte layer in the fracture limb ($44.8 \pm 2.2 \mu\text{m}$, $n=6$) but no change was observed in the contralateral paw ($20.4 \pm 2.3 \mu\text{m}$, $n=6$), as compared to controls ($16.5 \pm 1.4 \mu\text{m}$, $n=6$). These results suggest that distal tibia fracture leads to chronic keratinocyte proliferation and epidermal hyperplasia in the skin of the hindpaw.

3.2. Fracture increased expression of cytokine protein in hindpaw keratinocytes

We previously observed that distal tibia fracture is accompanied by an increase in IL-1 β , IL-6, and TNF- α mRNA and protein levels in the hindpaw skin [42;43;55]. Immunohistochemistry was thus performed to identify which cells express these mediators at 4 weeks post-fracture (Figs. 2, 3 and 4). Although few IL-1 β expressing cells were detected in sham operated control rats (Fig. 2 upper panel), skin sections from fracture rats demonstrated that IL-1 β protein was strongly up-regulated in keratinocytes, especially in the deeper layers of the epidermis (Fig. 2 lower panel). Quantification of the IL-1 β -positive cells in hindpaw skin revealed a 248% increase in IL-1 β positive cells in the fracture limb (55 ± 8.34 cells/high-power field (HPF), $n=5$), as compared to skin from sham operated controls (22.2 ± 2.35 cells/HPF, $n=5$) (Fig. 6A). Interestingly, we also observed the transformation of the basal layer of keratinocytes, which in control animals had the morphology that is typical of resting keratinocytes (Fig. 2, upper left panel), into a thicker layer of multiple cells (Fig. 2, lower left panel) that is indicative of activated keratinocytes after fracture.

Fig. 3 shows representative confocal microscopy images for IL-6 protein in keratinocytes in hindpaw skin sections from control rats and from the hindpaws of fractured limbs. These photomicrographs illustrate that IL-6 is expressed at a low level in the epidermis prior to fracture, but is dramatically up-regulated 4 weeks after fracture. The highest expression was again localized in the deeper epidermal layers. Quantification study revealed a 213% increase in IL-6 positive cells in the fracture limb (44.8 ± 4.18 cells/HPF, $n=5$), as compared to skin from sham operated controls (21 ± 3.81 cells/HPF, $n=5$) (Fig. 6B).

Fig. 4 presents representative immunostaining for TNF- α . We observed co-expression of TNF- α and keratin in the epidermis, with a clear increase in TNF- α expression in keratinocytes in all viable layers of the epidermis after fracture. There was a 269% increase in TNF- α positive cells in hindpaw skin in the fracture limb (79.75 ± 8.71 cells/HPF, $n=4$), as compared to skin from sham operated controls (29.75 ± 1.44 cells/HPF, $n=4$) (Fig. 6C).

3.3 Fracture increased expression of NGF- β protein expression in hindpaw skin

Enhanced levels of the neurotrophin NGF- β were observed in our previous studies in tissues from the limbs of rats 4 weeks post fracture [30;42]. We therefore determined whether NGF- β was induced in keratinocytes after fracture. Although NGF- β expression was low in control rats, skin sections from fracture rats demonstrated a robust up-regulation of NGF- β in keratinocytes across the epidermal layer (Figure 5). Fig. 6D shows a 163% increase in NGF- β positive cells in hindpaw skin in the fracture limb (76.4 ± 7.28 cells/HPF, $n=5$), as compared to skin from sham operated controls (47 ± 4 cells/HPF, $n=5$).

3.4 Intraplantar cytokine injection dose-dependently induced mechanical allodynia in intact rats

The nociceptive and vascular effects of IL-6 and TNF- α local injection were examined in normal intact (non-fractured) rats to complement existing data showing sensitization after IL-1 β and NGF injection [31]. Intraplantar IL-6 injection dose-dependently induced prolonged mechanical allodynia lasting between 1 and 48 hours after a dose of 10 ng and between 0.5 and 72 hours after a dose 60 ng (Fig. 7A). There was an insignificant elevation of hindpaw temperature at 1 hour post-injection (60ng, Fig. 7B), and a significant increase in hindpaw skin thickness at 1 hour post-injection with the highest dose of IL-6 (60 ng, Fig. 7C).

Intraplantar injections of TNF- α dose-dependently induced mechanical allodynia at 6 hours post-injection with a dose of 10 ng and prolonged mechanical allodynia lasting between 1 and 72 hours after a dose of 70 ng (Fig. 8A). There were significant increases in hindpaw temperature lasting between 6 and 24 hours after a dose of 70 ng (Fig. 8B), and significant increases in hindpaw edema lasting between 1 and 3 hours after a dose of 10 ng and 1 and 6 hours after a dose of 70 ng of TNF- α (Fig. 8C).

4. Discussion

Cytokines are a heterogeneous group of soluble small polypeptides produced by lymphocytes and macrophages, as well as a variety of other types of cells. They are key players in cell signaling events underlying inflammation and pain associated with tissue injury [10;48;51]. Likewise NGF is produced by and affects a number of immune, inflammatory and neuronal cell types. One of the key functions of NGF is to initiate and maintain pain hypersensitivity, a hallmark symptom of inflammation [28;38]. Using a rat tibia fracture model of CRPS, we recently demonstrated elevated levels of IL-1 β , IL-6, TNF- α and NGF- β in the hindpaw skin of the fractured limb. In the case of IL-1 β , TNF- α and NGF- β we have provided pharmacologic data demonstrating these mediators contribute to the nociceptive sensitization characterizing this model [31;42;43;55]. No increase in macrophages, neutrophils or T lymphocytes was observed in the hindpaw skin of the fracture animals, thus leukocytes are an unlikely source for these inflammatory mediators [55]. Because keratinocytes, the main constituent cell type of the epidermis, are key cells in the initiation and maintenance of inflammation in several skin diseases [1;2;13;35], we hypothesized that the over-expression of cytokines and NGF observed in the hindpaw skin after fracture was due to the proliferation and activation of keratinocytes.

The present study demonstrated that tibia fracture leads to the chronic proliferation of keratinocytes in the epithelial layer, and that these activated keratinocytes are the primary source for the increased expression of the cutaneous inflammatory mediators IL-1 β , IL-6, TNF- α , and NGF- β observed at 4 weeks post-fracture. It has been long recognized that cultured keratinocytes can be activated by stress (ultraviolet irradiation or osmotic shock), infection or microbial products (lipopolysaccharide), or IL-1 cytokine [2;15]. Activated keratinocytes can express IL-1 β , IL-6, TNF- α [3] and NGF- β [50] in vitro but little data addresses keratinocyte expression of these inflammatory mediators in vivo. We recently reported the up-regulation of IL-1 β mRNA and protein in keratinocytes after fracture [31], but the current study is the first to demonstrate in vivo keratinocyte expression of IL-6, TNF- α , and NGF- β . How fracture can cause keratinocytes to chronically over-express inflammatory cytokines and NGF is unknown, but we postulate that exaggerated sensory neuronal signaling plays a critical role.

Neurogenic inflammation occurs when sensory neurons are activated by noxious or electrical stimuli, causing the co-release of the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) from the distal nerve terminals innervating the skin. SP acts on endothelial NK1 receptors in the microvasculature to induce protein extravasation and edema. Using intravenous injections of radiolabeled immunoglobulin in CRPS patients, Dutch

investigators demonstrated that 48-hour protein extravasation was increased by an average of 50% in the affected hand [33]. Cutaneous microdialysis techniques have been used to demonstrate that electrically evoked protein extravasation responses are facilitated in the skin of CRPS patients, striking evidence that exaggerated neurogenic inflammation occurs in the CRPS extremity [53]. Furthermore, serum levels of SP are elevated in CRPS patients [47]. Similarly, SP expression is increased in the sensory neurons innervating the injured hindlimb and serum SP levels are elevated at 4 weeks after tibia fracture in rats [54].

Microdialyzed SP-evoked protein extravasation responses are exaggerated in the CRPS limb [27] and we have observed in fracture rats that intravenous SP perfusion causes an enhanced extravasation response and immediate paw edema in the injured limb but not on the contralateral side [19]. We also have observed increased NK1 receptor mRNA and protein expression in the hindpaw skin at 4 weeks post-fracture. The increase in NK1 receptor expression was localized by confocal microscopy to the endothelial cells and keratinocytes in the injured hindpaw skin and we suspect that facilitated neuro-cutaneous signaling contributes to the CRPS-like sequelae of fracture [54]. We tested this hypothesis by treating fracture rats with an NK1 receptor antagonist over the first 4 weeks post-fracture. Blocking SP signaling attenuated the development of edema, warmth, spontaneous extravasation, and nociceptive sensitization in the CRPS model [18]. Regarding the mechanism for fracture induced keratinocyte activation and expression of inflammatory mediators, numerous in vitro experiments have shown that SP is a powerful initiator of keratinocyte activation, causing increased cellular proliferation [36] and expression of IL-1 β , TNF- α , and NGF- β [8;11;49;52]. Collectively, these data support the hypothesis that fracture causes chronic up-regulation of the SP NK1 receptor in keratinocytes in the injured hindlimb, resulting in the over-expression of inflammatory mediators that contribute to the development of nociceptive sensitization, edema, and warmth after trauma.

Suction blister fluid from the affected limb in CRPS patients exhibits elevated IL-6 and TNF- α protein levels, but no increase in IL-1 β is observed, unlike our immunostaining results in the fracture rats [21;22]. This paradoxical situation resembles the results observed in the skin of psoriasis patients where IL-1 β levels from skin blister fluid are undetectable but immunohistochemistry in skin sections from psoriatic lesions demonstrate increased IL-1 β immunostaining on the plasma membrane and intracellular compartment of epidermal cells [40]. These conflicting results suggest that IL-1 β may not be readily detectable in the interstitial fluid obtained from skin blisters, but is never-the-less upregulated in the epidermis.

In the current study it was observed that IL-6 and TNF- α administered into the skin of the hindpaw cause long-lasting mechanical allodynia and some of the vascular effects characteristic of CRPS. Previously we demonstrated that intraplantar injections IL-1 β and NGF- β also induce dose-dependent allodynia and minor vascular changes [31]. This results support the hypothesis that the analgesic effects of cytokine and NGF inhibitors in the fracture model are due to local inhibitory effects on inflammatory mediator signaling in the skin. Our results are consistent with the reports of others that the direct injection of IL-1 β , IL-6, TNF- α and NGF into skin causes nociceptive sensitization, especially to mechanical stimuli [10;14;44;57;58]. Though the specific mechanisms have not yet been fully described, it is notable that intraneural injection of IL-1 β and TNF- α causes pain [59], and that IL-1 β and TNF- α stimulate discharges in primary afferent neurons [34;46].

The acute peripheral effects of IL-1 β following intraplantar administration could be mediated directly by excitation of nociceptive small-diameter A δ and C fibers as suggested by Fukuoka et al., who demonstrated activation of somatosensory fibers within 1 min after intraplantar IL-1 β injection [16]. Notably, nociceptors are IL-1 β sensors, and IL-1 β acts in a p38 mitogen-activated protein kinase (MAPK)-dependent manner, to increase its excitability by increasing

tetradotoxin-resistant (TTX-r) voltage-gated sodium channel currents [5]. TNF- α also increases TTX-r sodium currents in nociceptor DRG neurons via p38 [23] possibly contributing to its pro-nociceptive actions. Finally, NGF may directly sensitize peripheral nociceptor via binding to its high-affinity NGF receptors (trkA receptors) in nociceptive terminals [38], and this sensitization may start within minutes and persist for several hours (early response) [12;29;37;58].

Besides the positive effects of IL-1 β , IL-6, and TNF- α on nociception, intraplantar administration of these mediators modestly increased hind paw warmth and edema. Similarly, we previously observed that pentoxifylline, a global cytokine inhibitor, reduced nociceptive sensitization and some vascular changes in the rat fracture CRPS model [55]. However, selective anti-IL-1 β or anti-TNF- α therapy reduced nociceptive sensitization with limited effects on the vascular changes characteristic of the rat fracture CRPS model [31;42;55]. Likewise, anti-NGF had no effect on the warmth and actually increased the edema present in rat hindpaws at 4 weeks post-fracture [43]. Therefore, altering local levels of just one of the potentially many signaling molecules involved in the pathogenesis of these inflammatory vascular changes seems to have little efficacy in preventing these changes. In addition to the sum of cytokine and neurotrophin effects, there is strong evidence that the vascular manifestations of CRPS may rely on the effects of primary afferent neurotransmitters like SP and CGRP which cause edema and temperature elevation when administered cutaneously [6;54]. Though largely unexplored, the increased vascular permeability and edema also could be mediated by numerous other mediators like histamine, serotonin, prostaglandins, leukotrienes, and tryptase released from mast cells which may have enhanced activity in the skin of CRPS patients [22]. Supporting this hypothesis are observations that chronically elevated TNF- α and NGF levels enhance mast cell recruitment and mediator release in other systems [7;24].

Taken together it was observed that in normal animals resting keratinocytes produce limited amounts of IL-1 β , IL-6, TNF- α , and NGF- β , and keratinocyte proliferation appears to be at a low level. However, fracture triggers keratinocyte activation, proliferation, and the generation of excess amounts of these mediators, which are capable of inducing skin hyperplasia and long-lasting nociceptive sensitization, warmth and edema. These effects may involve direct excitation of nociceptive fibers and possibly indirect or autocrine stimulation of keratinocytes to produce even larger quantities of inflammatory mediators. It is noteworthy that several reports measuring fluid from skin suction blisters in humans with CRPS have demonstrated elevated levels of inflammatory cytokines, perhaps due to enhanced production in resident skin cells [17;21;22;32]. Further studies on the role of keratinocytes and their derived mediators may provide insights into the underlying mechanisms involved in CRPS and pave the way for novel therapeutic strategies.

Summary

Tibia fracture causes local activation of skin keratinocytes leading to expression of pronociceptive inflammatory mediators that could potentially contribute to complex regional pain syndrome.

Acknowledgments

This work was funded by Department of Veteran Affairs, Veterans Health Administration, Rehabilitation Research and Development Service grant F4516I. The work was also supported by NIH award GM079126. The authors do not have financial or other relationships that might lead to conflict of interest.

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Figure 1.

Epidermal keratinocyte proliferation and the thickness of the epidermal keratinocyte layer in the hindpaw skin at 4 weeks post-fracture. (A) Co-immunostaining for keratin (a keratinocyte marker, red) and BrdU (a cell proliferation marker, green) in hindpaw skin sections from control rats and from the hindpaws ipsilateral (Fx-ipsilateral) and contralateral (Fx-contralateral) to tibia fracture at 4 weeks post-injury. Rats were treated by BrdU for 8 d before skin harvest. Scale bar = 50 μm, the same magnification was used in all images within the figure. (B) Quantification of BrdU positive (BrdU+) cells in the epidermis demonstrated a 350% increase in BrdU+ cells in the fracture limb. (C) Measurements of epidermal thickness identified a 280% increase in epidermal thickness in the fracture limb. *** $p < 0.001$ for Fx-ipsilateral vs. control rat values, and ### $p < 0.001$ for Fx-ipsilateral vs. Fx-contralateral rat values.

Figure 2.

Fluorescence photomicrographs of IL-1 β protein in the hindpaw skin at 4 weeks post-fracture. Top panels are from a normal control rat, bottom panels are from the fracture hindpaw. Co-immunostaining for keratin, (a keratinocyte marker, green) and IL-1 β (red) in the epidermis demonstrates dramatically increased IL-1 β protein expression in keratinocytes after fracture, preferentially in the basal layer of the epidermis. Scale bar = 20 μ m, the same magnification was used in all images within the figure.

Figure 3.

Fluorescence photomicrographs of IL-6 protein in the hindpaw skin at 4 weeks post-fracture. Top panels are from a normal control rat, bottom panels are from the fracture hindpaw. Co-immunostaining for keratin, (a keratinocyte marker, green) and IL-6 (red) in the dermis demonstrates dramatically increased IL-6 protein expression in keratinocytes after fracture, preferentially in the basal layer of the epidermis. Scale bar = 20 μ m, the same magnification was used in all images within the figure.

Figure 4.

Fluorescence photomicrographs of TNF- α protein in the hindpaw skin at 4 weeks post-fracture. Top panels are from a normal control rat, bottom panels are from the fracture hindpaw. Co-immunostaining for keratin, (a keratinocyte marker, green) and TNF- α (red) in the dermis demonstrates dramatically increased TNF- α protein expression in keratinocytes in all viable layers of the epidermis after fracture. Scale bar = 20 μ m, the same magnification was used in all images within the figure.

Figure 5.

Fluorescence photomicrographs of NGF- β protein in the hindpaw skin at 4 weeks post-fracture. Top panels are from a normal control rat, bottom panels are from the fracture hindpaw. Co-immunostaining for keratin, (a keratinocyte marker, green) and NGF- β (red) in the dermis demonstrates dramatically increased NGF- β protein expression in keratinocytes after fracture, preferentially in the basal layer of the epidermis. Scale bar = 20 μ m, the same magnification was used in all images within the figure.

Figure 6. Quantification of IL-1 β (A), IL-6 (B), TNF- α (C), and NGF- β (D) positive keratinocytes in the hindpaw skin at 4 weeks post-fracture. ** $p < 0.01$ for Fx-ipsilateral vs. control rat values.

Figure 7.

Dose-dependent effects of IL-6 intraplantar injection on hindpaw von Frey thresholds (A), temperature (B), and thickness (C) in normal control rats (n = 6 per injection cohort). (A) Intraplantar IL-6 injection dose-dependently induced mechanical allodynia lasting between 1 and 48 hours after a dose of 10 ng and between 0.5 and 72 hours after a dose 60 ng in intact rats. (B) There was an insignificant elevation of hindpaw temperature at 1 hour post-injection with the highest dose of IL-6 (60ng). (C) There was a significant increase in hindpaw thickness at 1 hour post-injection with the highest dose of IL-6 (60ng). An insignificant elevation of hindpaw thickness at 1 hour post-injection with the dose of 10ng was also observed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 8.

Dose-dependent effects of TNF- α intraplantar injection on hindpaw von Frey thresholds (A), temperature (B), and thickness (C) in normal control rats. (A) Intraplantar TNF- α injection induced mechanical allodynia at 6 hours post-injection with a dose of 10 ng and prolonged mechanical allodynia lasting between 1 up to 72 hours after a dose of 70 ng. (B) There were significant increases in hindpaw temperature lasting between 6 and 24 hours after a dose of 70 ng of TNF- α . (C). There were significant increases in hindpaw edema lasting between 1 and 3 hour after a dose of 10 ng and 1 and 6 hours after a dose of 70 ng of TNF- α . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.