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Activation of Cutaneous Immune Responses in Complex Regional Pain Syndrome

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Abstract: The pathogenesis of complex regional pain syndrome (CRPS) is unresolved, but tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are elevated in experimental skin blister fluid from CRPS-affected limbs, as is tryptase, a marker for mast cells. In the rat fracture model of CRPS, exaggerated sensory and sympathetic neural signaling stimulate keratinocyte and mast cell proliferation, causing the local production of high levels of inflammatory cytokines leading to pain behavior. The current investigation used CRPS patient skin biopsies to determine whether keratinocyte and mast cell proliferation occur in CRPS skin and to identify the cellular source of the up-regulated TNF-α, IL-6, and tryptase observed in CRPS experimental skin blister fluid. Skin biopsies were collected from the affected skin and the contralateral mirror site in 55 CRPS patients and the biopsy sections were immunostained for keratinocyte, cell proliferation, mast cell markers, TNF-α, and IL-6. In early CRPS, keratinocytes were activated in the affected skin, resulting in proliferation, epidermal thickening, and up-regulated TNF-α and IL-6 expression. In chronic CRPS, there was reduced keratinocyte proliferation, leading to epidermal thinning in the affected skin. Acute CRPS patients also had increased mast cell accumulation in the affected skin, but there was no increase in mast cell numbers in chronic CRPS.

***Perspective:** The results of this study support the hypotheses that CRPS involves activation of the innate immune system, with keratinocyte and mast cell activation and proliferation, inflammatory mediator release, and pain.*

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***Key words:** Complex regional pain syndrome, pain, immunology, keratinocytes, mast cells.*

Complex regional pain syndrome (CRPS) is a painful, disabling, and often chronic condition with an estimated 50,000 new cases in the United States each year.[7] Evidence from CRPS patients and human volunteers suggests that both sensory primary afferent C-fibers and sympathetic neurons function aberrantly in CRPS, resulting in vascular symptoms, trophic changes, and pain.[4,5,30] Additional studies measuring cytokines such as tumor necrosis factor–alpha (TNF-α) and interleukin-6 (IL-6) have demonstrated that these mediators are elevated in the experimental skin blister fluid of CRPS patients,[11,17,19,31] as is tryptase, a marker for mast cells capable of releasing a host of nociceptive and vasoactive mediators.[21] Furthermore, TNF-α levels are increased in CRPS skin biopsies when measured by enzyme immunoassay and in CRPS-affected hands scintigraphically imaged with radiolabeled anti-TNF-α antibody.[1,24]

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Population-based studies indicate that distal limb fracture is the most common cause of CRPS[7,37] and we have developed a distal tibia fracture model in rats and mice that closely parallels the regional changes observed in early CRPS, including hind limb allodynia, unweighting, increased spinal Fos-immunoreactivity, increased skin temperature, edema, increased spontaneous protein extravasation, and periarticular bone loss.[12,13,29,34,35] Using the rat fracture model of CRPS, we have shown that facilitated substance P (SP) and calcitonin gene–related peptide (CGRP) signaling from sensory C-fibers,[44] as well as norepinephrine released from sympathetic nerve terminals, induces inflammation and pain sensitization by the activation of neuropeptide and adrenergic receptors on the surface of keratinocytes, causing keratinocytes to proliferate and secrete high levels of inflammatory cytokines and nerve growth factor, inflammatory mediators that contribute to pain behavior in this CRPS model.[14,26,27,29,34,35,39,45] The release of SP from C-fibers also caused mast cell accumulation and degranulation, further contributing to pain behavior in the rat fracture model.[28] Based on these data, we postulate that posttraumatic up-regulated neuropeptide and sympathetic adrenergic signaling induces keratinocyte and mast cell activation, proliferation, and inflammatory mediator expression in CRPS skin. This investigation examined patient skin biopsies to determine whether keratinocyte and mast cell proliferation occurs in CRPS skin and to identify the cellular sources of the up-regulated TNF-α, IL-6, and tryptase previously observed in CRPS experimental skin blister fluid and skin biopsies.[11,19,21,31]

Methods

Subjects and Clinical Data Collection

The study protocols were approved by the respective local institutional review boards at Murdoch University and at the University of Mainz. Thirty-seven women (67%) and 18 men (33%) with CRPS were enrolled after giving written informed consent. The subjects' average age was 49.8 $\pm$ 1.8 years, with a range of 20 to 72 years. Inclusion criteria included 1) meeting the new International Association for the Study of Pain clinical diagnostic criteria for CRPS at the time of biopsy,[16] 2) unilateral symptoms in the hand or foot, allowing use of the contralateral limb for control biopsy, and 3) no recent glucocorticoid or bisphosphonate treatments (as these drugs potentially inhibit cytokine expression). Patient demographics and clinical data were recorded, including age, gender, CRPS duration and etiology, involved limb, pain medications, numerical 11-point pain score, allodynia, and limb temperature. Allodynia was tested by applying 3 or 4 light strokes with a small brush to the affected skin and asking patients if this evoked a normal or abnormal sensation. If the sensation was described as abnormal, the patient was asked to give a qualitative description of the sensation. Descriptions of the brushing as uncomfortable or painful were regarded as allodynia. Patients were further tested by applying 3 or 4 light strokes to the skin with the tip of a paper clip, and if the patient described that stimulation as uncomfortable or painful the response was also categorized as allodynia. Limb temperature was also measured over 5 separate sites in the affected skin and over the mirror-image area of the contralateral healthy limb, using an infrared skin thermometer (Tempett IR Thermometer; Somedic Sales AB, Hörby, Sweden; or Fluke IR Thermometer No. 561; Fluke, Norwich, Norfolk, United Kingdom), and the difference between the averaged values in the CRPS-affected limb and the contralateral limb was calculated.

Tissue Processing and Microscopy

Under local anesthesia, full-thickness 3-mm skin punch biopsies were obtained from the bilateral hands or feet of 55 CRPS patients. The biopsies were collected from the same location bilaterally in each patient, so that the normal (unaffected) side control biopsy was the mirror image of the affected side skin biopsy. Biopsies were fixed in Zamboni's fixative (FD NeuroTechnologies, Columbia, MD) for 4 hours at 4°C, then rinsed with .1 M phosphate buffer (pH 7.4) and 50% ethanol followed by embedding in TissuePrep2 paraffin (Fisher Scientific, Waltham, MA). Following embedding, 8- to 10-μm slices were cut, mounted onto slides (Tru Scientific, Bellingham, WA), deparaffinized in xylene, and hydrated through graded alcohols to distilled water. Paraffin sections were antigen-retrieved by IHC-Tek Epitope Retrieval Solution (IHC World, Woodstock, MD) steaming for 40 minutes, then cooled to room temperature. Sections were permeabilized and blocked with phosphate-buffered saline containing 10% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and .3% Triton X-100, followed by exposure to primary antibodies overnight at 4°C in phosphate-buffered saline 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 phosphate-buffered saline and incubated with fluorophore-conjugated secondary antibodies against the immunoglobulin of the species from which the primary antibody was generated. After 3 washes, the sections were counterstained with DAPI (Thermo Fisher Scientific, Waltham, MA) solution (.25 μg/mL) to identify nuclei and mounted with anti-fade mounting medium (Invitrogen Molecular Probes, Eugene, OR). Images were obtained using confocal microscopy (Zeiss LSM/510 Upright 2 photon; Carl Zeiss, Thornwood, NY) and stored on digital media. With regard to primary antibodies, rabbit anti-human IL-6, 1:600 (Abcam, Cambridge, UK), mouse monoclonal to human mast cell tryptase Ab-2, clone AA1, 1:1,000 (Thermo Fisher Scientific), mouse monoclonal antibody to Pan keratin, 1:75 (Thermo Fisher Scientific), and rabbit monoclonal antibody to Ki-67, clone SP6, 1:1,000 (Thermo Fisher Scientific) were used. Double labeling immunofluorescence was performed with donkey anti-mouse IgG (1:500)