Published in final edited form as:

Pain. 2012 February ; 153(2): 366–372. doi:10.1016/j.pain.2011.10.032.

The Complement Component C5a Receptor Mediates Pain and Inflammation in a Postsurgical Pain Model

De-Yong Liang^{1,2,3}, XiangQi Li², Xiaoyu Shi², Yuan Sun^{1,2}, Peyman Sahbaie^{1,2}, Wen-Wu Li^{1,2}, and J David Clark^{1,2}

Department of Anesthesiology VAPAHCS and Stanford University School of Medicine, CA 94304

¹Department of Anesthesiology, Veterans Affairs Palo Alto Health Care System, Palo Alto, CA

²Department of Anesthesiology, Stanford University School of Medicine Stanford, CA

Abstract

The complement system is an important part of innate immunity. Complement activation generates a set of effector molecules with diverse biological functions. C5a is a crucial terminal component of the complement cascade. Several reports suggest C5a can support nociceptive sensitization and inflammation in various models including models of incisional pain. However, information concerning the differential effects of C5a on specific modalities of nociception, the role of C5a in supporting neutrophil infiltration, secondary nociceptive mediator generation, and the location of the relevant populations of C5a receptors supporting incisional sensitization are needed. In these studies we utilized C5a receptor null mice (C5aR^{-/-}) and matched controls to study nociceptive changes after hind paw incision. Heat hyperalgesia and mechanical allodynia were measured for 4 days after incision. We also followed hind paw edema, wound area neutrophil infiltration using the myeloperoxidase assay, and IL-1 β and NGF levels using both ELISA and immunohistochemical techniques. The main findings were: 1) Heat versus mechanical nociceptive sensitization after incision were differentially reduced in C5aR^{-/-} mice with thermal sensitization affected throughout the post-incisional period but mechanical sensitization affected only at later time points; 2) Edema developed after incision in wild type mice but only slightly and transiently in C5aR^{-/-} mice, and 3) Deletion of C5aR blocked IL-1 β and NGF production near the wound site. These findings demonstrate the complement system component C5a is a novel biomarker and mediator associated with postsurgical nociceptive processing. C5aR may provide a novel target for the control of pain and inflammation after surgery.

Keywords

Incision; Complement; C5a Receptor; Pain; Inflammation; Nerve Growth Factor; IL-1 β ; Mouse

1. Introduction

Since its discovery over 100 years ago, the complement cascade has been recognized as an important effector arm of the innate system of immunity. Many byproducts generated during

© 2011 International Association for the Study of Pain. Published by Elsevier B.V. All rights reserved.

³Corresponding author: VAPAHCS and Stanford University, 3801 Miranda Ave, Palo Alto, CA 94304, Phone: 650.493.5000 ext 67184, Fax: 650.852.3423, dyliang@stanford.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

complement activation are mediators of inflammation [11; 12; 42]. The complement system consists of more than 30 soluble and membrane-bound proteins. Complement activation is initiated by the tissue binding of one or more molecules of the classical, alternative, or mannose-binding lectin pathways[22]. Eventually, all three pathways converge, with subsequent activation of complement proteins C3 and C5 in the terminal cascade. The small complement fragments generated during complement activation, C3a and C5a, are known anaphylatoxins that induce several biological responses. They act through their respective G-protein coupled receptors to exert a range of biological effects. Uncontrolled complement activation can lead to excess tissue inflammation and damage, which occurs in many immune-complex-mediated diseases such as rheumatoid arthritis [21; 44]. Recently, the role of complement system, especially C5a, in pain processing has been gaining attention [9; 14; 19; 24; 34; 41]. For example, C5a receptor blockade decreased pain behaviors in inflammatory, neuropathic and postoperative pain models [9; 14; 19; 41]. Electrophysiological studies demonstrate that intraplantar injection of C5a activates and sensitizes cutaneous nociceptors, especially heat-sensitive C-fibers, but not mechanico-sensitive A fibers [19].

Postoperative pain is an expected consequence of most surgeries. One of the underlying causes of this pain is the surgically induced production and release of a variety of inflammatory mediators constituting an “inflammatory soup” at the wound site [9; 10; 17; 18; 31; 32]. Elevation of the levels of many cytokines has been reported in the wounds of rodents and humans including Interleukin-1(IL-1) β , IL-6, tumor necrosis factor alpha (TNF α), granulocyte colony stimulating factor (G-CSF), macrophage-inflammatory protein 1 (MIP-1 α), keratinocyte-derived cytokine, nerve growth factor(NGF) and others[3; 9; 10; 31; 43; 45; 46; 49]. Several of these mediators are produced in large amounts by the epidermis after incision [10; 18; 31; 32]. Blockade of C5aR partially prevents cytokine production in models of incision and arthritis [9; 44]. Recently our laboratory demonstrated that the C5aR antagonist PMX-53 dramatically reduced the production of several cytokines in the skin surrounding hind paw incision with parallel reductions in nociceptive sensitization [9]. Our knowledge base is, however, deficient in that there has been little exploration of the modalities of nociceptive sensitization supported by C5a after incision, the requirement for intact C5a signaling has not been rigorously studied with respect to IL-1 β and NGF production though these are two of the best established wound area nociceptive mediators, and the role of C5a in supporting wound area inflammation and immune cell infiltration has not been well examined.

Relevant to these deficiencies in our fund of knowledge, the results of our present experiments show that C5a signaling would have differential effects on heat versus mechanical nociceptive signaling, that edema and neutrophil infiltration would rely largely on C5a signaling, that the generation of both IL-1 β and NGF require C5a signaling, and that the epidermis is an area key to C5a in supporting sensitization after incision.

2. Materials and Methods

2.1. Animals

All experimental protocols were reviewed and approved by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee before the initiation of work. All protocols conform to the guidelines for the study of pain in awake animals as established by the International Association for the Study of Pain. Male C5a receptor deficient mice(C.129S4-C5ar1^{tm1cge}/J, stock number 006854) on a BALB/C background and BALB/C control mice, 8-9 weeks old were purchased from The Jackson Laboratory (JAX; Bar Harbor, ME). Mice were housed 5 per cage, and maintained on a 12-h light/dark cycle and an ambient temperature of 22 $\pm$ 1°C, with food and tap water available ad libitum.

2.2. Surgical Preparation

Paw incision in mice was performed as described previously [9; 10; 31]. Briefly, mice were anesthetized with isoflurane delivered via a nose cone. After sterile preparation of the right hindpaw, a 0.5cm longitudinal incision was made through skin and fascia of the plantar surface of the foot with a number 11 scalpel blade. The incision was started 0.2cm from the proximal edge of the heel and extended distally. The underlying muscle was elevated with curved forceps, leaving the muscle origin and insertion intact. After wound hemostasis, the skin was apposed with a 6.0 nylon mattress suture and the wound was covered with antibiotic ointment. In some experiments, control mice without incision underwent a sham procedure that consisted of anesthesia, antiseptic preparation, and application of the antibiotic ointment without an incision.

2.3. Pain behaviors

2.3.1. Heat Hyperalgesia—Paw withdrawal response latencies to noxious heat stimulation were measured using the method of Hargreaves et al. [15] as we have modified for use with mice [29]. In this assay, mice were placed on a temperature-controlled glass platform (29 °C) in a clear plastic enclosure similar to those described above. After 30 min of acclimation, a beam of focused light was directed towards the same area of the hindpaw as described for the von Frey assay. A 20s cutoff was used to prevent tissue damage. In these experiments, the light beam intensity was adjusted to provide an approximate 10s baseline latency in control mice. Three measurements were made per animal per test session separated by at least one minute.

2.3.2. Mechanical allodynia—Mechanical nociceptive thresholds were assayed using von Frey filaments according to a modification of the “up-down” algorithm described by Chaplan et al.[8] as described previously [29; 30]. Mice were placed on wire mesh platforms in clear cylindrical plastic enclosures of 10cm diameter and 30cm in height. After 20 minutes of acclimation, fibers of sequentially increasing stiffness with initial bending force of 0.2 gram were applied to the plantar surface of the hindpaw adjacent to the incision, just distal to the first set of foot pads and left in place 5 sec with enough force to slightly bend the fiber. Withdrawal of the hindpaw from the fiber was scored as a response. When no response was obtained, the next stiffer fiber in the series was applied in the same manner. If a response was observed, the next less stiff fiber was applied. Testing proceeded in this manner until 4 fibers had been applied after the first one causing a withdrawal response allowing the estimation of the mechanical withdrawal threshold using a curve fitting algorithm [36].

2.4. Hindpaw edema

Paw thickness from the dorsal to ventral hindpaw surface was measured by a laser sensor technique as previously described by Clark and et al. [9]. Briefly, mice were first anesthetized by exposure to isoflurane. Each hindpaw was then held in turn against a flat surface, above which was affixed a laser device capable of triangulating thickness with a precision of 0.01 mm (model 4381 Precicura; Limab, Goteborg, Sweden). Paw thickness was measured over the third metatarsal at a point 1-2 mm distal to the most distal aspect of the incision. For each animal, three measurements were made of both the incised and non-incised hindpaws.

2.5. Tissue harvest and protein isolation

Mice were euthanized immediately after behavioral measurement at the time points specified in the figures. The skin tissue surrounding incision with approximate 1.5mm margins was excised, and skin specimens were placed into phosphate-buffered saline

containing a protease inhibitor cocktail (Roche Complete, Roche Diagnostics, Mannheim, Germany), and frozen at -80°C until analysis. Likewise, deeper tissues including fascia and muscle from the wounds were harvested and handled separately. For use these samples were first cut into small pieces with microscissors then disrupted using a Polytron Device (Brinkmann Instruments Inc, Westbury, NY). The samples were then centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was carefully pipetted into a fresh 1.5ml tube, which was the material used for protein analysis. Protein concentration was evaluated with a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

2.6. IL-1 β and NGF assays

IL-1 β and NGF levels were quantified using enzyme-linked immunosorbent (ELISA) assays according to the kit manufacturer's instructions, and used the provided standards of mouse IL-1 β (eBioscience, San Diego, CA), and NGF (Chemicon, Billerica, MA). The lower limits of detection of the levels IL-1 β and NGF were 5pg/mL and 3.9pg/mL, respectively. The optical density of the reaction product was read on a microplate reader at 450 nm, and values were normalized per mg of tissue assayed.

2.7. Myeloperoxidase (MPO) assay

MPO activity was measured as a biochemical index of neutrophil recruitment in the wound edge samples as described previously by our lab [10; 31]. Excised skin samples were washed in PBS and placed in 1 ml 50 mM potassium phosphate-buffer solution with 0.5% hexadecyl trimethyl ammonium bromide (Sigma Chemical Co., St. Louis, MO) and 5 mM EDTA. The samples were then homogenized as above and centrifuged at 12,000 rpm at 4°C . MPO activities in the supernatants were assayed using a peroxidase assay kit (Anaspec, San Jose, CA) along with supplied standards according to the manufacturer's instructions. The data were expressed as MPO activity in mU per mg protein in the supernatant samples.

2.8. Immunohistochemical Analysis

We previously reported our methods for the immunohistochemical analysis of incised mouse paw skin [9; 10]. For these analyses mice were sacrificed using carbon dioxide asphyxiation, followed by intra-cardiac perfusion of 20 ml 0.9% NaCl followed by 20 ml of 10% neutrally buffered formalin. Hind paws were then removed and incubated in 10% buffered formalin for an additional 24 hours. After overnight decalcification, tissue was processed for paraffin sectioning in automated fashion (Tissue Tek VIP, Miles Scientific, Naperville, IL). Following embedding, 6.0 μm slices were made then placed on slides and incubated for 20 min at 55°C to improve adherence. Paraffin was removed with graded xylenes then sections were re-hydrated in ethanol. Blocking of these sections took place overnight at 4°C in Tris-buffered saline containing 5% dry milk, followed by exposure to the primary antibodies against IL-1 β (Santa Cruz Biotechnology, Santa Cruz, CA) or NGF (Abcam, Cambridge, MA) overnight at 4°C in milk- Tris-buffered saline. Sections were then be rinsed and transferred to milk-Tris-buffered saline containing fluorescein conjugated secondary antibodies against the primary antibodies, 1:300-1:500 (Jackson ImmunoResearch Laboratories, West Grove, PA) and incubated for 1 hour. After washing, coverslips were applied. Confocal laser-scanning microscope was carried out using a Zeiss LSM/510 META microscope (Thornwood, NY). Control experiments included incubation of slices in primary and secondary antibody free-solutions, both of which lead to low intensity non-specific staining patterns in preliminary experiments.

2.9. Statistical analysis

The results are expressed as mean $\pm$ SEM. The data for mechanical sensitivity, heat sensitivity, edema and cytokine expression were analyzed by two-way analysis of variance

(ANOVA) followed by Bonferroni post-hoc test for multiple comparisons. For simple comparisons of 2 groups, two tailed Student's t-testing was employed. P values less than 0.05 were considered significant (Prism 4, GraphPad Software, La Jolla, CA).

3. Results

3.1. C5aR disruption suppresses heat hyperalgesia after paw incision

Heat hyperalgesia is characteristic of peri-incisional tissues. Paw incision induced significant heat hyperalgesia lasting the full observational period of 96 hours after incision ($F_{6, 140}=39.26$, $P<0.0001$), as shown in Figure 1A. However, C5aR null mice exhibited significantly less heat hyperalgesia over the full time course in comparison with wild-type mice (at least $P<0.05$). Wild-type mice had long-sustained heat hyperalgesia when compared with non-incisional controlateral side ($P<0.001$). The heat hyperalgesic sensitivities of non-incisional side were no significant change, similar to baseline in the full observational period between C5aR null- and wild-type mice ($P>0.05$).

3.2. C5aR disruption suppresses mechanical allodynia after paw incision

Mechanical allodynia is an important feature of human surgical wounds and is present in the rodent model of incisional pain for several days after incision [6; 35]. After making hind paw incisions in our experiments, the C5aR null mice exhibited a significant mechanical allodynia after incision comparable to wild type mice for the first 24 hours after incision ($P>0.05$). But the less sensitization was observed in this strain during the 48 to 96 hour time period (at least $P<0.05$) (Figure 1B).

3.3. C5aR disruption suppresses edema after paw incision

Edema is a common feature of tissue surrounding surgical wounds. We hypothesized that the C5a signaling pathway modulates this response as this molecule is closely associated with inflammation in many other tissues. Wild-type animals demonstrated a significant paw thickness increase after incision over the full time course of 96 hours ($F_{5, 120}=26.11$; $P<0.0001$), as shown in Figure 1C. However, C5aR null mice expressed only very slight edema within the first 24 hours of incision ($P<0.001$), which rapidly diminished in comparison with the controlateral side ($P>0.05$). Incision-induced swelling was different between the strains for the 6-48 hour time period after incision ($F_{3, 120}=189.48$; $P<0.0001$).

3.4. IL-1 β and NGF Production after paw incision

IL-1 β and NGF are mediators crucial to the modulation of post-incisional nociceptive sensitization [3; 7; 39; 45; 46; 49]. Furthermore, recent evidence indicates that these nociceptive mediators likely function in both skin and deeper tissues to support pain after incision[5]. In our studies, IL-1 β and NGF levels in peri-incisional skin and deeper tissues (muscle and fascia) were significantly increased in time-dependent manner after incision in wild-type animals as displayed in Figures 2 and 3. However, no significant alterations in the levels of these mediators were detected in C5aR null animals in either skin or muscle during the observational period.

3.5. Localization of IL-1 β , NGF, and C5aR in peri-incisional tissues

To further characterize the IL-1 β , NGF, and C5aR expression in peri-incisional tissues, we assessed the distribution of these molecules in skin of C5aR deficient mice using immunohistochemical techniques. Figure 4 demonstrates that in wild type mice IL-1 β expression was increased in epidermis, dermis and muscle after incision compared with control tissue in which there was only very weak immunostaining at 24 hours after incision.

However, IL-1 β immunoactivity in peri-incisional tissues was not detectable in C5aR null animals.

Figure 5A and B demonstrate that NGF was increased in the epidermal, dermal and layers after incision in wild-type animal, but not in C5aR null animals. Likewise, NGF was also increased in muscle after incision in wild-type animals, but not in C5aR null mice (Figure 5C and D).

3.6. Neutrophil immigration into the wound site

Previous work from our laboratory and others demonstrated that infiltrating neutrophils are a potential source of cytokines and NGF in incisional wounds [9; 31]. C5a plays an important role in the recruitment and activation of neutrophils in some settings [25; 44]. Here, we sought to determine whether C5aR was essential for the recruitment of neutrophils to the wound area after incision. The myeloperoxidase assay (MPO) was used as an index of neutrophil immigration. The data in Figure 6 show that hind paw incision led to a significant rise in MPO activity in skin and muscular tissues 2-48 hours after incision in wild-type animals. Likewise, MPO levels were increased in C5aR null mice at the time point of 2 hours after incision and decreased more rapidly in the null mice.

4. Discussion

Recent studies have shown that the C5a/C5aR system is involved in pain processing [9; 19; 23; 41]. Earlier results from our laboratory based on the effects of a C5aR antagonist showed that C5aR blockade reverses mechanical sensitization after incision in a delayed manner, and that some additional aspects of the inflammatory response such as the generation of several cytokines could be reduced as well [9]. To this point, the roles of C5a in pain processing have been evaluated mainly by using C5a receptor antagonists or by following C5 mRNA changes [9; 14; 19; 25; 41; 44]. In the present study, we utilized the power inherent in the use of receptor (C5aR) null mutant mice to extend our existing understanding of the functions of C5a in incisional wounds. Some of the key observations were, 1) that C5a signaling is essential for heat hyperalgesia throughout the post-incisional period whereas the contributions of C5a to mechanical sensitization are somewhat delayed, 2) that C5a signaling is essential for the generation of both the archetypical cytokine IL-1 β closely associated with incisional sensitization [18; 32; 43], and NGF, a neurotrophin particularly important for heat sensitization after incision [3; 39; 45; 49], 3) that C5a supported expression of mediators is prominent in the epidermis of skin near incisions, but includes deeper tissues as well, and 4) that additional dimensions of the inflammatory process initiated by incision such as edema and neutrophilic infiltration rely critically on C5a signaling.

The effects of C5aR antagonists on mechanical nociceptive sensitization have been evaluated in inflammatory, peripheral neuropathic and paw incision pain models [9; 14; 41]. However, the role of C5a in heat sensitization has been less well described. Though our animal testing paradigm is not identical to the human testing protocols it is noteworthy that heat pain sensitivity was strongly associated with postoperative pain and analgesic consumption [1]. Our recent studies revealed local injection of PMX-53, a C5aR antagonist, better suppressed heat pain sensitivity than mechanical allodynia in mouse incisional model [20]. We observed in additional previous work that recombinant C5a added to skin-nerve preparations sensitized C-fibers to heat stimuli [19]. Consistent with those observations were calcium imaging studies using DRG cells demonstrating that responses to capsaicin, a TRPV1 agonist, were augmented in the presence of C5a; the TRPV1 receptor-channel has well established heat sensing properties [19]. Direct effects of C5a to sensitize nerve fibers to mechanical stimuli were not observed. In the present studies we compared heat and

mechanical sensitization after incision in the same animals. While no differences in baseline thresholds were found before incision, heat sensitization was reduced throughout the post-incisional period, whereas the C5aR null mutant mice displayed similar mechanical sensitization with wild-type mice for the first 24 hours after incision (Figures 1A and B). Thus it may be reasonable to conclude either that C5a works through different pathways to support heat as opposed to mechanical sensitization, or possibly that despite C5aR deletion there are sufficient alternative pathways supporting mechanical sensitization acutely after incision such that C5a effects are not discernable for at least 24 hours.

One possibility for the prominent role of C5a in supporting heat hyperalgesia throughout the post-incisional period is that C5a is working both directly on nociceptors and indirectly through NGF to stimulate heat-sensitive C-nociceptors. It was already mentioned that C5a and TRPV1 channels expressed on these fibers may interact. The expression of C5a receptors on primary sensory neurons has been established [19]. Our studies demonstrate a time-dependent production of NGF after injury as assessed using ELISA in peri-incisional tissues consistent with the results of others using alternative techniques [3; 45; 46; 49]. In our mouse incisional model, these NGF levels peak at about 48 hours after incision, and are completely dependent on intact C5aR signaling. While this time course of NGF expression in comparison to the time course of thermal sensitization makes it difficult to conclude that NGF drives all thermal sensitization, substantial evidences points to its involvement. NGF predominantly mediates heat hyperalgesia through sensitization of heat response c-fibers although it may mediate some component of mechanical sensitization as well [2; 16; 37; 38; 40]. Systemic or local injection of NGF induces heat hyperalgesia within 1 hour, but the onset of mechanical hyperalgesia is delayed for several hours [26]. Ruiz, et al. reported that endoneural administration of NGF produces heat hyperalgesia but does not affect the mechanical threshold measured using von Frey hairs [38]. In vitro study of cutaneous nociceptors in rat skin suggest NFG sensitizes heat-sensitive nociceptor, but not mechanical or cold sensitive nociceptors [37]. In NGF overexpressing mice the endogenous increase in NGF levels dramatically enhances heat sensitivity of C-fibers[40]. Lastly, when anti-NGF antibody was administered to rats undergoing hind paw incision, heat but not mechanical sensitization was reduced [3].

In addition to supporting NGF production, C5aR mediates IL-1 β production in the wound site induced by incision which is a mediator linked to nociceptive sensitization [18; 32; 43]. Our previous work demonstrates that blockade of C5a Receptor with PMX53 reduces IL-1 β production in peri-incisional skin [9]. The current work using the C5aR null animals confirms the observations in skin, and extends the observations to deeper tissues. Moreover, blockade of IL-1 β production with inhibitors of caspase blocked both heat and mechanical nociceptive sensitization after incision [32]. Importantly, IL-1 β production in peri-incisional tissue was linked to edema in these studies, suggesting roles for this cytokine beyond nociception. It is felt that IL-1 β can serve as a “master” inflammatory mediator stimulating the production of multiple additional inflammation-related molecules [4; 33]. Thus C5a control of IL-1 β production may have consequences on wound biology well beyond those immediately due to the changing levels of IL-1 β itself.

Complement split products including C5a have known properties as chemoattractants for neutrophils and other inflammatory cell types. Neutrophils produce an abundance of mediators including neurotrophins and cytokines which could contribute to nociceptive sensitization in the surgical wounds. Data provided in these studies and in our previous work show that neutrophils present in peri-incisional tissues produce both IL-1 β and NGF [10]. In the current experiments we show that in the acute post-incisional period myeloperoxidase (MPO) activity, an index of neutrophil accumulation, is initially similar in wild type and C5aR null mice. However, this enhanced MPO activity is somewhat more transient in the

C5aR null animals, indicating that the contribution of neutrophil-generated mediators may be less in the C5aR null animals at later time points. The slight reduction in MPO activity in the wounds of C5aR null mutant mice would not seem to explain the essentially complete elimination of IL-1 β and NGF production in the null mutant mice. Thus C5aR signaling potentially stimulated the production of nociceptive mediators in both resident (keratinocytes) and infiltrating (neutrophils) cells. In fact, our immunohistochemical data displayed in Figure 4 demonstrate that keratinocytes in the epidermis stain strongly for the C5a receptor, the same cells which were observed to produce IL-1 β and NGF in incisional wounds. Previous work implicated C5a receptor signaling in keratinocytes as responsible for enhanced IL-6 production [13].

The data derived from these studies demonstrate that mediators regarded as nociceptive, i.e. NGF and IL-1 β , based on the results of many studies may act not only in the relatively well characterized superficial layers of tissue, but also in deeper structures including muscle. For example, our data are consistent with evidence demonstrating that keratinocytes are local sensory cells, which produce multiple cytokines and inflammatory mediators including C5a, NGF and IL-1 β after tissue injury [13; 27; 28]. These inflammatory mediators sensitize free ending fibers innervating the dermal and epidermal layers. However, both electrophysiological and behavioral studies have provided strong evidence for deeper tissues contributing to pain after incision [5]. For example, spontaneous nociceptor activity is more prominent in fibers innervating incised muscle than fibers innervating the incised overlying skin [48]. This activity may be attributable to the sensitization of groups III and IV afferent fibers. Indeed, incision of deep tissues is necessary for observation of prolonged guarding behavior in the hindpaw incisional pain model [47]. Our observations suggest that C5aR signaling may be relevant to both superficial and deep tissue signaling.

In summary, disruption of C5aR blocked nociceptive and inflammatory responses in a mouse postsurgical pain model. This effect was observed at earlier time points for heat as opposed to mechanical stimuli. Mechanisms explaining these effects include the elimination of direct C5a signaling, a reduction in the generation of two key inflammatory mediators, and a reduction in neutrophilic infiltration. These data suggest that C5aR antagonists may be viable analgesic agents for incisional pain and possibly other types of pain characterized by inflammation.

Acknowledgments

The authors do not have a conflict of interest.

This work is attributed to Department of Anesthesiology, Veterans Affairs Palo Alto Health Care System

Support: This work was supported by Research Grant of GMO79126 to (JDC) from the National Institutes of Health, Bethesda MD, USA.

References

- [1]. Abrishami A, Chan J, Chung F, Wong J. Preoperative pain sensitivity and its correlation with postoperative pain and analgesic consumption: a qualitative systematic review. *Anesthesiology*. 2011; 114(2):445–457. [PubMed: 21245740]
- [2]. Amann R, Schuligoi R, Herzeg G, Donnerer J. Intraplantar injection of nerve growth factor into the rat hind paw: local edema and effects on thermal nociceptive threshold. *Pain*. 1996; 64(2): 323–329. [PubMed: 8740610]
- [3]. Banik RK, Subieta AR, Wu C, Brennan TJ. Increased nerve growth factor after rat plantar incision contributes to guarding behavior and heat hyperalgesia. *Pain*. 2005; 117(1-2):68–76. [PubMed: 16061324]

- [4]. Basu A, Krady JK, Levison SW. Interleukin-1: a master regulator of neuroinflammation. *J Neurosci Res.* 2004; 78(2):151–156. [PubMed: 15378607]
- [5]. Brennan TJ. Pathophysiology of postoperative pain. *Pain.* 2011; 152(3 Suppl):S33–40. [PubMed: 21232860]
- [6]. Brennan TJ, Vandermeulen EP, Gebhart GF. Characterization of a rat model of incisional pain. *Pain.* 1996; 64(3):493–501. [PubMed: 8783314]
- [7]. Carvalho B, Clark DJ, Angst MS. Local and systemic release of cytokines, nerve growth factor, prostaglandin E2, and substance P in incisional wounds and serum following cesarean delivery. *J Pain.* 2008; 9(7):650–657. [PubMed: 18394968]
- [8]. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods.* 1994; 53(1):55–63. [PubMed: 7990513]
- [9]. Clark JD, Qiao Y, Li X, Shi X, Angst MS, Yeomans DC. Blockade of the complement C5a receptor reduces incisional allodynia, edema, and cytokine expression. *Anesthesiology.* 2006; 104(6):1274–1282. [PubMed: 16732100]
- [10]. Clark JD, Shi X, Li X, Qiao Y, Liang D, Angst MS, Yeomans DC. Morphine reduces local cytokine expression and neutrophil infiltration after incision. *Mol Pain.* 2007; 3:28. [PubMed: 17908329]
- [11]. Cole DS, Morgan BP. Beyond lysis: how complement influences cell fate. *Clin Sci (Lond).* 2003; 104(5):455–466. [PubMed: 12580763]
- [12]. Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science.* 1996; 271(5247):348–350. [PubMed: 8553069]
- [13]. Fayyazi A, Sandau R, Duong LQ, Gotze O, Radzun HJ, Schweyer S, Soruri A, Zwirner J. C5a receptor and interleukin-6 are expressed in tissue macrophages and stimulated keratinocytes but not in pulmonary and intestinal epithelial cells. *Am J Pathol.* 1999; 154(2):495–501. [PubMed: 10027407]
- [14]. Griffin RS, Costigan M, Brenner GJ, Ma CH, Scholz J, Moss A, Allchorne AJ, Stahl GL, Woolf CJ. Complement induction in spinal cord microglia results in anaphylatoxin C5a-mediated pain hypersensitivity. *J Neurosci.* 2007; 27(32):8699–8708. [PubMed: 17687047]
- [15]. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain.* 1988; 32(1):77–88. [PubMed: 3340425]
- [16]. Heppenstall PA, Lewin GR. Neurotrophins, nociceptors and pain. *Curr Opin Anaesthesiol.* 2000; 13(5):573–576. [PubMed: 17016360]
- [17]. Hu Y, Liang D, Li X, Liu HH, Zhang X, Zheng M, Dill D, Shi X, Qiao Y, Yeomans D, Carvalho B, Angst MS, Clark JD, Peltz G. The role of interleukin-1 in wound biology. Part I: Murine in silico and in vitro experimental analysis. *Anesth Analg.* 2010; 111(6):1525–1533. [PubMed: 20889942]
- [18]. Hu Y, Liang D, Li X, Liu HH, Zhang X, Zheng M, Dill D, Shi X, Qiao Y, Yeomans D, Carvalho B, Angst MS, Clark JD, Peltz G. The role of interleukin-1 in wound biology. Part II: In vivo and human translational studies. *Anesth Analg.* 2010; 111(6):1534–1542. [PubMed: 20889944]
- [19]. Jang JH, Clark JD, Li X, Yorek MS, Usachev YM, Brennan TJ. Nociceptive sensitization by complement C5a and C3a in mouse. *Pain.* 2010; 148(2):343–352. [PubMed: 20031321]
- [20]. Jang JH, Liang D, Kido K, Sun Y, Clark DJ, Brennan TJ. Increased local concentration of complement C5a contributes to incisional pain in mice. *J Neuroinflammation.* 2011; 8:80. [PubMed: 21736743]
- [21]. Jose PJ, Moss IK, Maini RN, Williams TJ. Measurement of the chemotactic complement fragment C5a in rheumatoid synovial fluids by radioimmunoassay: role of C5a in the acute inflammatory phase. *Ann Rheum Dis.* 1990; 49(10):747–752. [PubMed: 2241262]
- [22]. Kemper C, Atkinson JP. T-cell regulation: with complements from innate immunity. *Nat Rev Immunol.* 2007; 7(1):9–18. [PubMed: 17170757]
- [23]. Leinase I, Rozanski M, Harhausen D, Thurman JM, Schmidt OI, Hossini AM, Taha ME, Rittirsch D, Ward PA, Holers VM, Ertel W, Stahel PF. Inhibition of the alternative complement activation pathway in traumatic brain injury by a monoclonal anti-factor B antibody: a

- randomized placebo-controlled study in mice. *J Neuroinflammation*. 2007; 4:13. [PubMed: 17474994]
- [24]. Levin ME, Jin JG, Ji RR, Tong J, Pomonis JD, Lavery DJ, Miller SW, Chiang LW. Complement activation in the peripheral nervous system following the spinal nerve ligation model of neuropathic pain. *Pain*. 2008; 137(1):182–201. [PubMed: 18160218]
- [25]. Levine JD, Gooding J, Donatoni P, Borden L, Goetzl EJ. The role of the polymorphonuclear leukocyte in hyperalgesia. *J Neurosci*. 1985; 5(11):3025–3029. [PubMed: 2997412]
- [26]. Lewin GR, Mendell LM. Regulation of cutaneous C-fiber heat nociceptors by nerve growth factor in the developing rat. *J Neurophysiol*. 1994; 71(3):941–949. [PubMed: 8201434]
- [27]. Li WW, Guo TZ, Li XQ, Kingery WS, Clark JD. Fracture induces keratinocyte activation, proliferation, and expression of pro-nociceptive inflammatory mediators. *Pain*. 2010; 151(3):843–852. [PubMed: 20934254]
- [28]. Li WW, Sabsovich I, Guo TZ, Zhao R, Kingery WS, Clark JD. The role of enhanced cutaneous IL-1beta signaling in a rat tibia fracture model of complex regional pain syndrome. *Pain*. 2009; 144(3):303–313. [PubMed: 19473768]
- [29]. Li X, Angst MS, Clark JD. A murine model of opioid-induced hyperalgesia. *Brain Res Mol Brain Res*. 2001; 86(1-2):56–62. [PubMed: 11165371]
- [30]. Liang D, Li X, Lighthall G, Clark JD. Heme oxygenase type 2 modulates behavioral and molecular changes during chronic exposure to morphine. *Neuroscience*. 2003; 121(4):999–1005. [PubMed: 14580950]
- [31]. Liang D, Shi X, Qiao Y, Angst MS, Yeomans DC, Clark JD. Chronic morphine administration enhances nociceptive sensitivity and local cytokine production after incision. *Mol Pain*. 2008; 4:7. [PubMed: 18294378]
- [32]. Liang DY, Li X, Li WW, Fiorino D, Qiao Y, Sahbaie P, Yeomans DC, Clark JD. Caspase-1 modulates incisional sensitization and inflammation. *Anesthesiology*. 2010; 113(4):945–956. [PubMed: 20823759]
- [33]. Martinon F, Tschopp J. Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ*. 2007; 14(1):10–22. [PubMed: 16977329]
- [34]. Mika J. Modulation of microglia can attenuate neuropathic pain symptoms and enhance morphine effectiveness. *Pharmacol Rep*. 2008; 60(3):297–307. [PubMed: 18622054]
- [35]. Pogatzki EM, Raja SN. A mouse model of incisional pain. *Anesthesiology*. 2003; 99(4):1023–1027. [PubMed: 14508341]
- [36]. Poree LR, Guo TZ, Kingery WS, Maze M. The analgesic potency of dexmedetomidine is enhanced after nerve injury: A possible role for peripheral alpha2-adrenoceptors. *Anesth Analg*. 1998; 87(4):941–948. [PubMed: 9768799]
- [37]. Rueff A, Mendell LM. Nerve growth factor NT-5 induce increased thermal sensitivity of cutaneous nociceptors in vitro. *J Neurophysiol*. 1996; 76(5):3593–3596. [PubMed: 8930301]
- [38]. Ruiz G, Ceballos D, Banos JE. Behavioral and histological effects of endoneurial administration of nerve growth factor: possible implications in neuropathic pain. *Brain Res*. 2004; 1011(1):1–6. [PubMed: 15140639]
- [39]. Sahbaie P, Shi X, Guo TZ, Qiao Y, Yeomans DC, Kingery WS, Clark JD. Role of substance P signaling in enhanced nociceptive sensitization and local cytokine production after incision. *Pain*. 2009; 145(3):341–349. [PubMed: 19660865]
- [40]. Stucky CL, Koltzenburg M, Schneider M, Engle MG, Albers KM, Davis BM. Overexpression of nerve growth factor in skin selectively affects the survival and functional properties of nociceptors. *J Neurosci*. 1999; 19(19):8509–8516. [PubMed: 10493751]
- [41]. Ting E, Guerrero AT, Cunha TM, Verri WA Jr, Taylor SM, Woodruff TM, Cunha FQ, Ferreira SH. Role of complement C5a in mechanical inflammatory hypernociception: potential use of C5a receptor antagonists to control inflammatory pain. *Br J Pharmacol*. 2008; 153(5):1043–1053. [PubMed: 18084313]
- [42]. Walport MJ. Complement. First of two parts. *N Engl J Med*. 2001; 344(14):1058–1066. [PubMed: 11287977]

- [43]. Wolf G, Livshits D, Beilin B, Yirmiya R, Shavit Y. Interleukin-1 signaling is required for induction and maintenance of postoperative incisional pain: genetic and pharmacological studies in mice. *Brain Behav Immun*. 2008; 22(7):1072–1077. [PubMed: 18442892]
- [44]. Woodruff TM, Strachan AJ, Dryburgh N, Shiels IA, Reid RC, Fairlie DP, Taylor SM. Antiarthritic activity of an orally active C5a receptor antagonist against antigen-induced monarticular arthritis in the rat. *Arthritis Rheum*. 2002; 46(9):2476–2485. [PubMed: 12355496]
- [45]. Wu C, Boustany L, Liang H, Brennan TJ. Nerve growth factor expression after plantar incision in the rat. *Anesthesiology*. 2007; 107(1):128–135. [PubMed: 17585224]
- [46]. Wu C, Erickson MA, Xu J, Wild KD, Brennan TJ. Expression profile of nerve growth factor after muscle incision in the rat. *Anesthesiology*. 2009; 110(1):140–149. [PubMed: 19104181]
- [47]. Xu J, Brennan TJ. Comparison of skin incision vs. skin plus deep tissue incision on ongoing pain and spontaneous activity in dorsal horn neurons. *Pain*. 2009; 144(3):329–339. [PubMed: 19527922]
- [48]. Xu J, Brennan TJ. Guarding pain and spontaneous activity of nociceptors after skin versus skin plus deep tissue incision. *Anesthesiology*. 2010; 112(1):153–164. [PubMed: 19996955]
- [49]. Zahn PK, Subieta A, Park SS, Brennan TJ. Effect of blockade of nerve growth factor and tumor necrosis factor on pain behaviors after plantar incision. *J Pain*. 2004; 5(3):157–163. [PubMed: 15106128]

Figure 1.

Differences in nociceptive sensitivity and edema in wild-type (C5aR^{+/+}) and C5aR null mutant (C5aR^{-/-}) mice. Incisional side is indicated as solid line and closed circle (C5aR^{-/-}) or closed square (C5aR^{+/+}); and non-incisional contralateral side is indicated as dashed line and open circle (C5aR^{-/-}) or closed square (C5aR^{+/+}). (A) Thermal nociceptive sensitivity was assessed by paw withdrawal latency (seconds) in C5aR^{-/-} and C5aR^{+/+} mice. (B) Mechanical nociceptive sensitivity was assessed by paw withdrawal threshold (grams) in C5aR^{-/-} and C5aR^{+/+} mice. (C) Edema intensity was assessed by paw thickness (mm) in C5aR^{-/-} and C5aR^{+/+} mice. Values are displayed as the mean $\pm$ S.E.M., n = 6 for all groups, * p<0.05, ** p<0.01 or *** p<0.001 compared to the wild-type group; # p<0.05, ## p<0.01 or ### P<0.001 compared to the non incisional side.

Figure 2. IL-1 β production in incised skin (A) and deep tissue (B) in C5aR $^{-/-}$ and C5aR $^{+/+}$ mice. Values are displayed as the mean $\pm$ S.E.M., n = 6, *p<0.05, *** p<0.001 comparing the C5aR $^{-/-}$ with C5aR $^{+/+}$ groups.

Figure 3. NGF production in incised skin (A) and deep tissue (B) in C5aR^{-/-} and C5aR^{+/+} mice. Values are displayed as the mean $\pm$ S.E.M., n = 6, *p<0.05, ** p<0.01 or ***p<0.001 comparing the C5aR^{-/-} with C5aR^{+/+} groups.

Figure 4. IL-1 β immunohistochemistry in C5aR^{+/+} (upper panels) and C5aR^{-/-} mice (lower panels) 24 hours after paw incision. Images in columns A and B illustrate expression of IL-1 β in skin tissue from control and incised mice, respectively. Columns C and D illustrate expression of IL-1 β in muscle tissue from control and incised mice, respectively. The scale bar is 50 μ m.

Figure 5. NGF immunohistochemistry in C5aR^{+/+} (upper panels) and C5aR^{-/-} mice (lower panels) 24 hours after paw incision. Images in columns A and B illustrate expression of NGF in skin tissue from control and incised mice, respectively. Columns C and D illustrate expression of NGF in muscle tissue from control and incised mice, respectively. The scale bar is 50 μ m.

Figure 6. Myeloperoxidase activity in incised skin (A) and deeper tissue (B) of C5aR^{+/+} and C5aR^{-/-} mice. Data from C5a^{+/+} mice are represented by dashed lines and data from C5a^{-/-} mice are represented by solid lines.