Blockade of the Complement C5a Receptor Reduces Incisional Allodynia, Edema, and Cytokine Expression

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Background: **Activation of the complement system is one component of the inflammatory response. Various components of the complement system participate in killing foreign organisms, recruiting immune cells, enhancing edema, and stimulating cytokine formation. Complement-mediated enhancement of the inflammation surrounding surgical incisions may increase pain.**

Methods: **In these studies, the authors used a murine hind paw incisional model to study the role of the complement C5a receptor in supporting incisional inflammation. At baseline and at various time points after incision, they measured the effects of a highly selective C5a receptor antagonist on nociceptive thresholds, edema formation, and cytokine production in the skin surrounding the incision. They also measured changes in C5a receptor expression near the incisions.**

Results: **The once-daily injection of the C5a receptor antagonist AcF-[OPdChaWR] reduced mechanical allodynia and edema in the incised hind paw. A multiplexed cytokine assay revealed that 8 of the 18 cytokines examined showed significant increases in skin tissue abundance after incision. Distinct time courses for the patterns of elevation were seen, though some degree of resolution occurred for all cytokines within 96 h. For 7 of these 8 cytokines, the C5a receptor antagonist reduced the enhancement of expression. In addition, the authors found that the C5a receptor messenger RNA level increased 15-fold in the skin surrounding the incisions within 24 h and then slowly declined.**

Conclusions: **The tissue directly surrounding incisions in mouse hind paws undergoes large changes in the content of specific cytokines in addition to demonstrating edema and nociceptive sensitization. By blocking the receptor for one component of the complement system, C5a, all of these changes can be reduced. Complement receptor inhibitors may constitute a novel group of compounds useful in reducing the pain and swelling of surgical incisions.**

THE complement system has been shown to become activated in many acute, subacute and chronic injury states including sepsis, shock, blunt trauma, surgical trauma, burns, ischemia, arthritis, and others. Several excellent reviews tabulate the many demonstrated and proposed functions of the complement system.[1–3] Although generally helpful to the organism, in some instances, such as with autoimmune diseases or in sepsis,

activation of the complement system can have deleterious consequences. Many functions have been ascribed to the various components of the classic, alternative, and lectin complement pathways, but those most commonly invoked are enhancement of inflammation and direct attack of foreign organisms. Complement system activation is commonly measured by the quantification of activated complement protein "split products." Increases of these complement split products have been demonstrated in the serum and in fluids from the wounds of surgical patients.[4] The participation of the complement system in wound inflammation suggests that inhibition of the complement cascade might reduce the liberation of inflammatory mediators which support nociception like hydrogen ion, biogenic amines, eicosanoids, neuropeptides, and other molecules. Of special note, the skin surrounding incisions has been shown to contain increased amounts of several cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and others.[5,6] Each of these cytokines has been observed to support enhanced nociceptive sensitivity in various rodent models.[7–9] Although 18 cytokines were followed in these studies, our panel was not comprehensive of all known cytokines. Levels of these cytokines decrease as healing progresses in the 3–5 days after incisions.

Recently, there has been a focus on methods to reduce complement anaphylatoxin activity to reduce inflammation. These anaphylatoxins (C3a, C4a, and C5a) are active complement split products which have a number of biologic properties, including acting as chemoattractants and causing the degranulation of mast cells and polymorphonuclear cells.[10] These substances act through G protein–coupled cell-surface receptors to exert their biologic functions.[11,12] The existence of anaphylatoxin receptors, especially C5a receptors, on nonimmune cells including glia and central nervous system neurons has led to the hypothesis that these substances have functions in addition to ones strictly involving the immune system, including participation in neuroinflammation and neurodegenerative processes.[13–17] Therefore, efforts to develop C5a receptor antagonists have been intensive with the hope that these agents would demonstrate some utility in a number of different diseases characterized by inflammation.[18,19]

To reduce joint inflammation, Woodruff *et al.*[20] used a cyclic peptide C5a receptor antagonist in a rat model of inflammatory arthritis. The cyclic peptide compound AcF-[OPdChaWR] has been observed to block C5a-mediated effects for 24 h or more after single doses and is

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highly selective for its receptor while leaving the remainder of the complement system functional.[21,22] The compound is also resistant to metabolism in the gut and plasma.[23] These investigators documented significant reductions in joint swelling, gait disturbance, joint TNF-α and IL-6 levels, and other inflammation-related parameters when the inhibitor was given at doses of 1 or 3 $\cdot$ kg^{-1} $\cdot$ day^{-1}. Formal nociceptive testing was not attempted. The clear role of the complement system in inflammatory processes including those active in surgical wounds and the success of the use of a C5a receptor antagonist in a rat model of inflammatory arthritis led us to hypothesize that the same C5a receptor antagonist would reduce nociceptive sensitization, edema, and the generation of inflammatory mediators in mouse hind paw incisions.

Materials and Methods

Animal Use

All experimental protocols were reviewed and approved by Veterans Affairs Palo Alto Healthcare System (Palo Alto, CA) institutional animal care and use committee before the initiation of work. Male mice 12–14 weeks old and of the C57Bl/6J strain were kept under standard conditions with a 12-h light–dark cycle and were allowed food and water *ad libitum*. Mice were obtained from Jackson Laboratories (Bar Harbor, MA) and were kept in our animal facility a minimum of 1 week before use in experiments.

Hind Paw Incision

The hind paw incision model was used as modified for mice.[24] Briefly, mice were anesthetized using isoflurane. After sterile preparation, a 0.5-cm incision was made with a No. 15 scalpel blade on the plantar surface of one hind paw. This incision was sufficiently deep to divide the plantaris muscle longitudinally. After briefly holding pressure to stop any active bleeding, a single 6-0 nylon suture was placed, and antibiotic ointment was applied. Mice were then returned to their cages. None of the mice used in these experiments showed evidence of infection of these paws at the time of behavioral or biochemical assays (wound dehiscence or pus).

Nociceptive Testing

Mechanical allodynia was assayed using nylon von Frey filaments according to the "up–down" algorithm described by Chaplan *et al.*[25] as we have used previously to detect allodynia in mice.[26,27] In these experiments, mice were placed on wire mesh platforms in clear cylindrical plastic enclosures of 10 cm diameter. After 20 min of acclimation, fibers of sequentially increasing stiffness were applied to the center of the plantar surface of the right hind paw between the first set of foot pads and left in place 5 s. For incised animals, the fibers were placed directly on the wound edge. Withdrawal of the hind paw from the fiber was scored as a response. When no response was obtained, the next stiffest fiber in the series was applied to the same paw; if a response was obtained, a less stiff fiber was next applied. Testing proceeded in this manner until four fibers had been applied after the first one causing a withdrawal response allowing the estimation of the mechanical withdrawal threshold.[28]

Edema Measurement

Paw thickness was measured as described by Guo *et al.*.[29] Briefly, mice were first anesthetized by exposure to isoflurane. Each hind paw 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, two measurements were made of both the incised and nonincised hind paws. The ratio of these thickness measurements was used to compare mice.

Drug Administration

The C5a receptor antagonist AcF-[OPdChaWR] (Promics, Queensland, Australia) was dissolved in sterile 0.9% normal saline before use. The concentration was adjusted so that a 3-mg/kg dose could be administered subcutaneously in the skin of the back in a 100-μl volume. Mice received either this solution or the same volume of normal saline 1 h before incision and each day 1 h before behavioral testing, edema measurement, or tissue harvest.

Total RNA Isolation, Reverse Transcription, and Real-time PCR

To obtain skin samples for messenger RNA (mRNA) quantification, animals were first killed by carbon dioxide asphyxiation, and an ovular patch of full-thickness skin providing 1.5- to 2-mm margins surrounding the hind paw incisions was collected by dissection. Care was taken to include only dermal tissue. Each such patch contained approximately 12 mg tissue. Skin patches were placed in RNA preservative overnight then frozen at −80°C (RNA later; Qiagen, Valencia, CA). Before RNA purification samples were first homogenized using a Polytron device (Brinkman Instruments Inc., Westbury, NY) and then centrifuged for 10 min at 12,000g at 4°C. The supernatants were then processed using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. The purity and concentration of the purified RNA was determined spectrophotometrically. Subsequently, complementary DNA (cDNA) was synthesized from this total RNA using random hexamer priming and a First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA).

Briefly, 1 μg total RNA was mixed with 4 μl 10x RT buffer; 8 μl MgCl$_2$, 25 mM; 4 μl DTT, 0.1 M; 1 μl RNasin; 2 μl SSII, 50 u/μl; 5 μl hexamers; and RNase-free water to 40 μl. Incubation was then performed at 42°C for 60 min followed by heat inactivation at 70°C. Finally, 1 μl RNase H was added to each reaction and incubated at 37°C for 20 min to degrade the RNA.

For real-time polymerase chain reaction (PCR), reactions were conducted in a volume of 4 μl using the Sybr Green I master kit (PE applied Biosystems, Foster City, CA). Briefly, 2 μl of a mixture of 2x Sybr Green and C5a receptor primers (forward: gtcctgttcacgaccgtttt; reverse: acggtcggcactaatggtag) was loaded with 2 μl diluted cDNA template in each well. Mineral oil, 8 μl, was loaded in each well to prevent loss of solution. PCR parameters were 95°C, 5 min then (95°C, 30s → 60°C, 30s → 72°C, 60s) for 40 cycles. Melting curves were performed to document single product formation, and agarose electrophoresis confirmed product size. RNA, 18s, was used as an internal control. The 18s primers were purchased from Ambion (Austin, TX). Amplification kinetics for these products were found to be similar. The data from real-time PCR experiments were analyzed by the comparative C_t method. For these calculations, average C_t values from triplicate PCR reactions for C5a receptor were normalized to average C_t values for ones from 18s from the same cDNA preparations. The ratio of comparative expression of the C5a receptor gene between the samples from treated and untreated mice was calculated as $2^{-(\Delta\Delta Ct)}$, where C_t represents threshold cycle of PCR amplification, ΔC_t represents the difference in threshold cycle between target and reference, and $\Delta\Delta Ct$ represents the difference between ΔC_t (treated sample) and ΔC_t (untreated sample) for same gene. The RNA purification process and real-time PCR methods have been described in detail in our previous reports.[26,27]

Cytokine Analysis

For cytokine analysis, skin samples were first dissected as described in the previous section and placed immediately into ice-cold 0.9% NaCl containing a cocktail of protease inhibitors (Complete; Roche Applied Science, Indianapolis, IN). Approximately 750 μl inhibitor cocktail was used per 25 mg tissue. The tissue was then rapidly homogenized and clarified as described in the previous section. Supernatant fractions were kept frozen until use. An aliquot was subjected to protein assay (DC Protein Assay; Bio-Rad, Hercules, CA).

For the cytokine assays, Bio-Rad Bio-Plex 8 and 18-plex mouse cytokine analysis kits were used in conjunction with the Bio-Plex system array reader according to the manufacturer's directions. Samples were diluted 1:1 to 1:4 before incubation. All samples were run in duplicate in each assay, and at least two separate assays were performed for each set of samples. Standard curves for
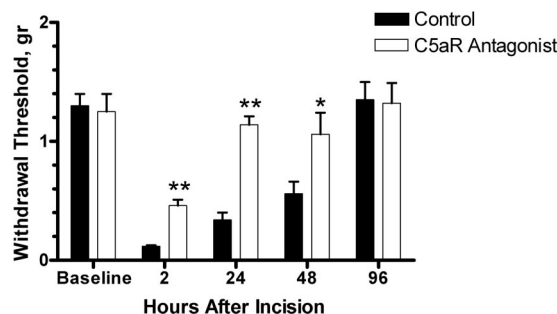

Fig. 1. Changes in mechanical nociceptive threshold after hind paw incision. Mice underwent either subcutaneous saline injections or injections of 3 mg/kg AcF-[OPdChaWR] 1 h before hind paw incision and daily thereafter 1 h before mechanical testing. Mechanical nociceptive thresholds were measured at various time points up to 96 h after incision. Both control and AcF-[OPdChaWR]–treated mice were also measured before incisions were made. Six mice were used in each group. Statistical comparisons were made between control and C5a receptor antagonist–treated mice at the indicated time points. Data are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$.

each of the analyzed substances were included in each run, and sample concentrations were automatically calculated.

Histologic Analysis

For histologic analysis of hind paw tissue, mice were sacrificed using carbon dioxide asphyxiation, which was followed by intracardiac perfusion of 0.9% NaCl as we have described previously.[30] Hind paws were then removed and incubated in 10% buffered formalin for 72 h before embedding in paraffin. After embedding, 6.5-μM slices were made and stained with hematoxylin and eosin. Sections were viewed using an Olympus BH-2 microscope and Spot RT digital imaging equipment (Diagnostic Instruments, Sterling Heights, MI).

Statistical Analysis

Analysis of repeated parametric measures was accomplished using a one-way analysis of variance followed by *post hoc* Dunnett testing or a two-way analysis of variance followed by Bonferroni testing. For simple comparisons of two means, two-tailed *t* testing was performed. A value of $P < 0.05$ was taken to be significant. All data are presented as mean ± SEM unless otherwise noted.

Results

Mechanical Allodynia after Hind Paw Incision

Mechanical allodynia is a feature of human surgical wounds as well as rat and mouse laboratory models of incisional pain. Because incisions are characterized by inflammation, we hypothesized that a C5a receptor antagonist would reduce mechanical allodynia after hind paw incision in mice. In figure 1, we display the time course of nociceptive thresholds in mice after hind paw incision. One group of mice received the long-acting C5a
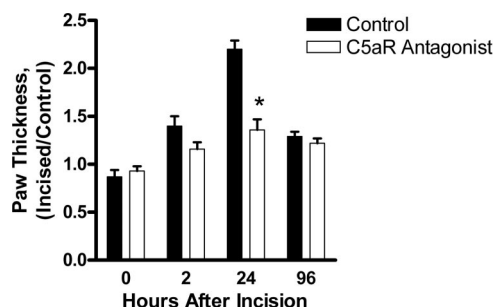
Fig. 2. Hind paw edema after incision. The ratio of incised *versus* contralateral hind paw thickness was calculated for eight mice in each experimental group at the time points indicated. Mice receiving C5a antagonist were injected 1 h before hind paw thickness measurement, whereas control mice received saline injections at the same times. Statistical comparisons were made between control and C5a receptor antagonist–treated mice at the indicated time points. Data are presented as mean ± SEM. * $P < 0.05$.

receptor antagonist AcF-[OPdChaWR] once per day, whereas the other group received only saline injections. At the 2-, 24-, and 48-h time points, the antagonist-treated animals displayed reduced primary mechanical allodynia. For both groups of mice, mechanical nociceptive thresholds had returned to near baseline levels by 96 h after the incisions were made. The C5a receptor antagonist did not affect baseline mechanical nociceptive thresholds.

Hind Paw Edema after Incision

Edema is a consistent feature of inflamed tissues. We hypothesized that the use of the C5a receptor inhibitor would reduce hind paw edema after incision. Figure 2 displays our data showing that hind paw edema reaches its peak value at approximately 24 h after incision and that a reduction in edema due to AcF-[OPdChaWR] administration can be demonstrated at that time point. Differences in hind paw thickness were not significantly different at the other time points. The C5a receptor antagonist did not affect baseline paw thickness.

Expression of C5a Receptor in Hind Paw Tissue after Incision

Because our C5a receptor antagonist was effective in reducing nociceptive sensitization and edema in the hind paw incisional model, we hypothesized that C5a receptor expression was increased in the skin of the incised hind paws. As shown in figure 3, levels of C5a receptor mRNA were greatly increased after incision and peaked with a 15-fold increase at the 24-h time point. This increase in expression had begun to resolve by the final time point. Peak C5a receptor mRNA levels approximate peak edema and peak C5a receptor antagonist effects, although it is clear that peak nociceptive sensitization occurs well before the peak in C5a mRNA has been reached (fig. 1).
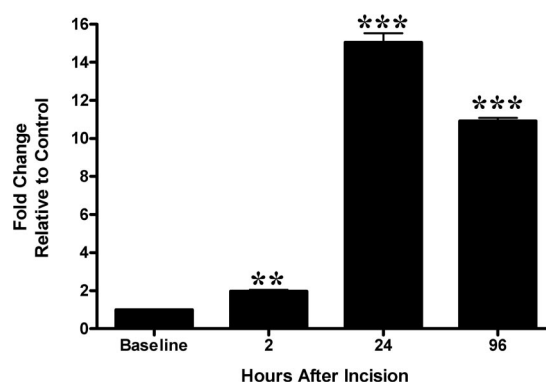
Fig. 3. Changes in C5a receptor messenger RNA (mRNA) levels in hind paw skin samples collected at the indicated time points after hind paw incision. Skin samples from four incised paws were analyzed in triplicate at each time point. 18s mRNA levels were measured as internal controls for the normalization of C5a receptor mRNA expression. Statistical comparisons were made between normalized baseline expression levels and those measured at the indicated time points. Data are presented as mean ± SEM. ** $P < 0.01$, *** $P < 0.001$.

Cytokine Expression in the Skin of Inflamed Hind Paws

Complement activation supports the overall inflammatory process, and the use of a C5a antagonist reduced joint space TNF-α and IL-6 levels in a model of inflammatory arthritis.[31] We hypothesized that the use of AcF-[OPdChaWR] would reduce the production of cytokines in the skin immediately surrounding the hind paw incisions. To accomplish these measurements, we used a multiplexed cytokine assay procedure. Table 1 shows

Table 1. Baseline Cytokine Levels in Hind Paw Skin

Molecule	Tissue Level
IL-1α	390 ± 44
IL-1β	9 ± 9
IL-2	1.7 ± 0.5
IL-3	4 ± 3
IL-4	0.5 ± 0.08
IL-5	0.9 ± 0.05
IL-6	113 ± 31
IL-10	13 ± 3
IL-12 p40	8 ± 3
IL-12 p70	29 ± 5
IL-17	29 ± 3
G-CSF	51 ± 9
GM-CSF	38 ± 11
IFN-γ	26 ± 9
KC	31 ± 2
MIP-1α	128 ± 15
RANTES	82 ± 5
TNF-α	102 ± 4

Table 1 presents the baseline levels of the 18 cytokines measured in these experiments. All measurements are given in terms of picograms cytokine per milligram hind paw tissue. Measurements represent tissues from 10 control hind paws, each measured in duplicate. Data are presented as mean ± SEM.

G-CSF = granulocyte colony-stimulating factor; GM-CSF, granulocyte–monocyte colony-stimulating factor; IFN-γ = interferon gamma; IL = interleukin; KC = keratinocyte-derived cytokine; MIP = macrophage inflammatory protein; RANTES = regulated on activation, normal T-cell expressed and secreted cytokine; TNF = tumor necrosis factor.

the baseline levels for 18 cytokines measured in the hind paw samples before incision. The assay we used had sufficient sensitivity to measure the basal levels for all of these substances, although for many cytokines, those levels were low.

Figure 4 provides the time course profiles for the 8 cytokines we observed to be present in statistically significantly increased levels at one or more time points after hind paw incision. The list of cytokines showing increased skin levels after incision included IL1-β; IL-2; IL-6; granulocyte colony-stimulating factor; keratinocyte-derived cytokine; macrophage inflammatory protein type 1α; regulated on activation, normal T-cell expressed and secreted cytokine; and TNF-α. The time course profiles for these cytokines varied somewhat, although for all substances, the enhanced levels had either returned to baseline or were beginning to diminish within 96 h of incision. Those cytokines that shared with nociceptive sensitization the property of being maximal at 2 h and resolving after that point included IL-2, IL-6, granulocyte colony-stimulating factor, keratinocyte-derived cytokine, and TNF-α. For all but one of the increased cytokines, macrophage inflammatory protein type 1α, the C5a receptor antagonist was observed to reduce the increased presence after incision. For none of the 18 cytokines tested were the baseline levels of cytokine significantly altered after C5a antagonist injection.

Leukocytic Infiltration of the Incisional Wound Area

Leukocytes present in areas of acute inflammation are capable of producing C5, C5a receptor, and cytokines.[32-35] We therefore undertook histologic experiments to determine the extent of leukocytic infiltration in tissue surrounding hind paw incisions. Figure 5a demonstrated the appearance of nonincised plantar skin, whereas C5b displays a micrograph of tissue adjacent to an incision. The 24-h time point was chosen because C5a receptor and cytokine expression were clearly increased at this time. A massive leukocytic infiltration was observed. The majority of these cells were neutrophils as expected.[36]

Discussion

We set out in these studies to address the role that the activated complement system might play in supporting incisional pain and to more fully identify inflammatory mediators present after incision. The principal findings of these studies were that (1) a C5a complement receptor antagonist reduced postincisional allodynia, (2) the C5a receptor antagonist reduced hind paw edema after incision, (3) C5a receptor expression was increased in the skin surrounding incisions, and (4) the C5a receptor antagonist reduced the levels of several inflammatory mediators present in incised skin. Although not necessarily identifying all cytokines, chemokines, and other inflammatory factors present in wounds, our experiments did identify a group of eight substances showing robustly increased levels after incision. Several of these substances, such as TNF-α, IL-1β, and IL-6, have putative roles in modulating pain.[7-9] Others, such as regulated on activation, normal T-cell expressed and secreted cytokine; keratinocyte-derived cytokine; and granulocyte colony-stimulating factor, do not have well-described functions related to pain at present.

Use of the C5a receptor antagonist AcF-[OPdChaWR] reduced cytokine levels after hind paw incision. For seven of the eight cytokines showing increased levels after incision, the antagonist-induced reductions were statistically significant, and levels of the eighth substance, macrophage inflammatory protein type 1α, trended toward lower levels. These decreases were up to 90% from the control levels. This generalized ability to reduce cytokine levels suggests that the function of C5a signaling in incisions is fundamental to the inflammatory process. Indeed, the broad expression of C5a receptors on macrophages, lymphocytes, keratinocytes, and other cells suggests that blocking C5a receptor activity might have a wide range of effects on wound inflammation.[37,38] Moreover, we measured a 15-fold increase in C5a receptor messenger RNA in the mouse wounds, again suggesting an important role for C5a in postincisional inflammation. One aspect of C5a function perhaps relevant to these effects is its chemoattractant properties. It could be hypothesized that blockade of C5a receptors would reduce leukocytic infiltration of the area, leading to a globally attenuated inflammatory response to incision. Although we have not yet attempted to measure C5a receptor–mediated influences on leukocytic infiltration in mice, disruption of C5a signaling reduces immune cell migration in models of joint inflammation.[20,39]

Our data allow some time course comparisons to be made. Nociceptive sensitization is maximal at 2 h after incision and slowly resolved thereafter, which is consistent with both the previously published mouse and rat data.[24,40] However, enhanced expression of C5aR lags this time course. In addition, our C5aR antagonist had significant effects before peak enhancement of C5aR mRNA. It should be recognized here that enhanced expression of functionally important molecules after painful stimuli often lag peak pain behaviors and nociceptive sensitization. For example, inhibitors of nitric oxide synthase, heme oxygenase, guanylate cyclase, and cyclic guanosine monophospate–dependent protein kinase all acutely reduce formalin-induced pain behaviors[41-44] but have peak mRNA levels 8–48 h later.[45] Increases in protein expression are similarly delayed.[45] Presumably, preexisting protein is responsible for some of the C5aR-mediated effects at early time points in our experiments.
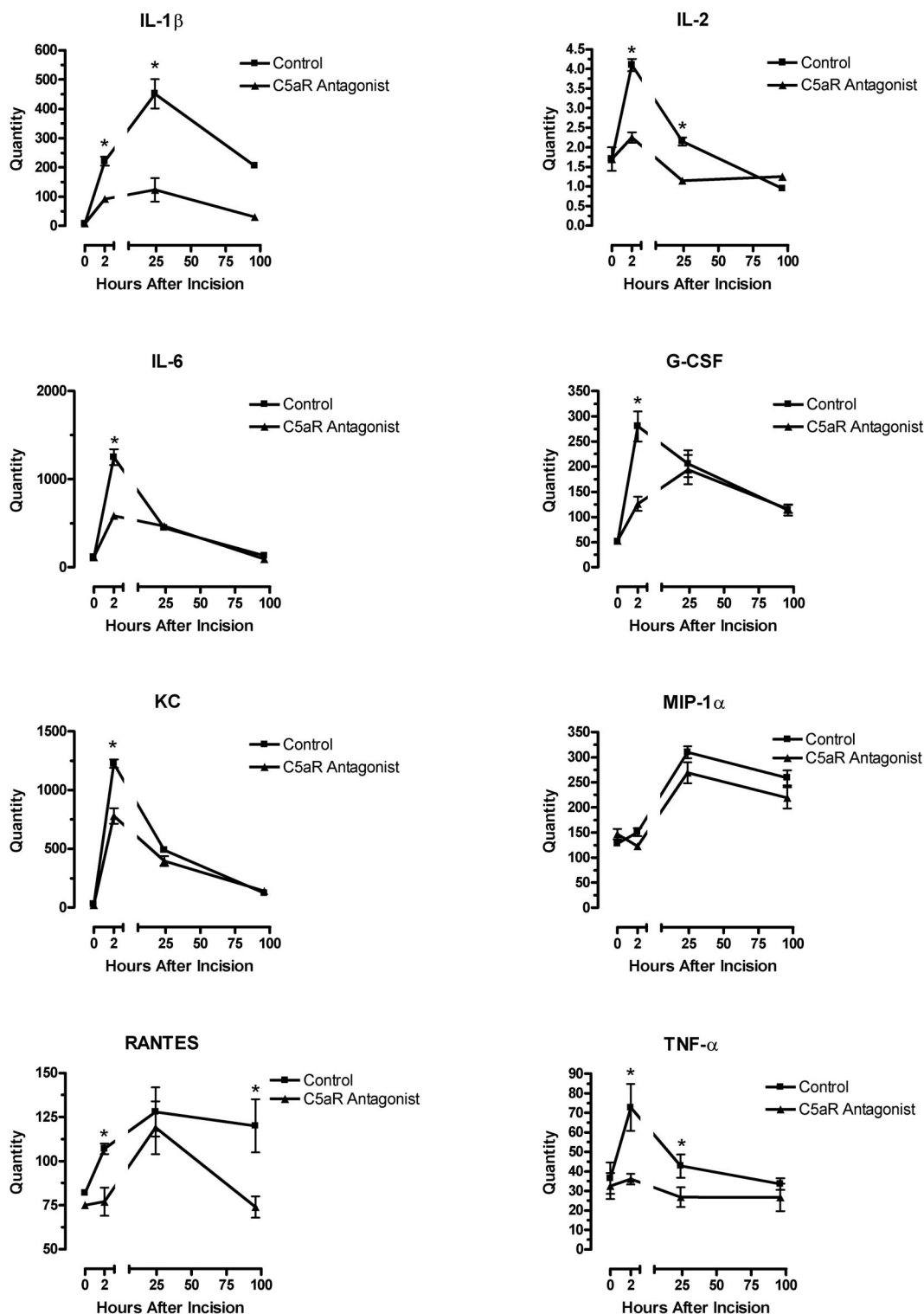
Fig. 4. Cytokine time course profiles both under control and C5a antagonist treatment conditions. The cytokines displayed in this figure are the group of eight cytokines observed to have statistically increased cytokine levels at one or more time points over the 4 days after hind paw incision. Each panel is labeled with the identity of the cytokine having been measured. G-CSF = granulocyte colony-stimulating factor; IFN = interferon; IL = interleukin; KC = keratinocyte-derived cytokine; MIP = macrophage inflammatory protein; RANTES = regulated on activation, normal T-cell expressed and secreted cytokine; TNF = tumor necrosis factor. For these experiments, the skins from 10 paws were used for cytokine analysis at each time point for each of the two treatment conditions. *Filled squares* represent data from control animals, and *filled triangles* represent data from C5a receptor antagonist–treated mice. Statistical comparisons were made between control and C5a receptor antagonist–treated mice at the indicated time points. Data are presented as mean ± SEM. * $P < 0.05$.
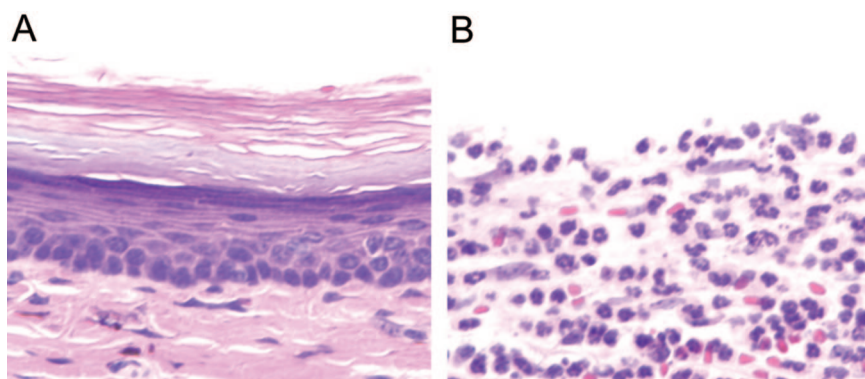
Fig. 5. Histologic appearance of mouse hind paw tissue. *A* displays a micrograph of nonincised hind paw skin from the plantar surface after hematoxylin and eosin staining. A normal stratified epithelial architecture was observed. *B* is a micrograph of tissue directly adjacent to an incision. This tissue was harvested 24 h after the incision was made. The section is remarkable for the massive predominantly neutrophilic infiltration of leukocytes and scattered erythrocytes. Both images were obtained using a 40× objective.

However, it is not until 24 h after incision that the C5aR antagonist reverses allodynia to near baseline, which is a time point showing peak C5aR mRNA levels. Therefore, the increased expression of a gene may be consistent with participation in a nociceptive signaling pathway even if the increase in expression is somewhat delayed. It must be borne in mind that nociceptive sensitization after incision is a complex process with both central and peripheral components.[46] Complement system activation is unlikely to explain the nociceptive time course completely. It is hoped that future studies more specifically examining the time courses of C5aR expression on resident *versus* infiltrating cells will allow us to determine which C5aR expressing sells are involved in nociceptive sensitization *versus* other complement-mediated functions.

In theory, the complement cascade could be targeted at any one of a number of levels depending on the clinical end point that is sought. The only clinically available agent available at present directly modulating the complement system is C1 inhibitor, a pooled blood product.[47,48] This agent is very effective in treating hereditary and acquired angioedema. Although pooled C1 inhibitor has shown some promise in treating other situations characterized by complement activation, including sepsis, pancreatitis, and other vascular leak syndromes,[49-51] the lack of small molecule C1 inhibitors and concerns over the immunologic consequences of inhibiting the classic arm of the complement system at a very proximal step have limited the use of this strategy.

Relatively little systematic investigation of the inflammatory mediators present in human or rodent incisional wounds has been undertaken. One study following a limited number of cytokines (IL-1α, IL-1β, IL-6, and TNF-α) after incisions in mice observed changes in these cytokines over a time course similar to that which we observed.[6] Another study again using incisions in mice identified a rapid peak then decline in IL-10 production,[52] although in our studies, no change in IL-10 levels was detected. Recently, Bryan *et al.*[5] used a murine wound model to show transient increases in some of the same cytokines we identified as increased in incisional wounds using enzyme-linked immunosorbent assay techniques, *e.g.*, IL-1β, IL-6, and TNF-α. These authors did not, however, find increased levels of other mediators we found to be significantly increased, including keratinocyte-derived cytokine; regulated on activation, normal T-cell expressed and secreted cytokine; and macrophage inflammatory protein type 1α or IL-2. Differences in the location of the wounds, depth of incisions, methods of measurement, strain of mice used, and lack of surgical closure in the other group's mice may explain some of these differences. Van der Laan *et al.*[53] provided one of the few reports attempting to compare cytokine production and cellular infiltration in incised human skin *versus* human skin involved in infection or overlying fractures. In these studies, the authors identified granulocyte infiltration of the surgical wound edges but did not find altered levels of TNF-α, IL-1α, or IL-8 in the few hours after incision using immunohistochemical techniques. Although not involving the epidermis, Kalff *et al.*[54] provided observations of rapid increase of IL-6, IL-1β, and TNF-α after incision of the human intestine muscularis externa layer. That cytokine profile was again similar to the results obtained in the current study. In none of the aforementioned studies were measurements of nociception attempted, nor was the modulation of these cytokine levels with medications explored. A unique example of the sequestration of endogenous incisional TNF-α with injected TNF-α receptor protein did not reduce the thermal or mechanical sensitization caused by the hind paw incision of rats.[55]

Although the characteristics of the inflammatory response and the specific role of the complement system are poorly understood for incisional lesions, work is at a much farther advanced stage for inflammatory arthritis. Using a model of collagen-induced arthritis, Grant *et al.*[39] documented joint infiltration of neutrophils, T cells, and macrophages in addition to the accumulation of the cytokines TNF-α and IL-1β. However, in C5a receptor–deficient mice, all indices of inflammation were sharply reduced. In the same set of studies, mice deficient in the C3a receptor did not demonstrate these reductions in joint space inflammatory markers. These observations are consistent with the known roles of C5a as the most potent chemoattractant of the anaphylatoxins and a mol-

ecule that can stimulate the release of cytokines and other inflammatory mediators from macrophages and other cells.[56] In other experiments, the cyclic peptide C5a receptor antagonist AcF-[OPdChaWR] was used by Woodruff *et al.*[20] to reduce gait disturbance, joint swelling, and joint space cytokine levels in a rat model of inflammatory arthritis at a dose of $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$. This compound has been shown to be effective for at least 24 h after administration, which allowed us to use once-per-day dosing in our studies.[22] The results from our incisional model are remarkably similar to the results from the arthritis models, particularly with respect to reductions in swelling and cytokine generation.

Despite the clear role of pain related to acute inflammation in many common clinical situations, we have a limited number of useful tools specifically targeting inflammatory mechanisms. The use of nonsteroidal agents is common, although the inhibition of cyclooxygenase-1 and cyclooxygenase-2 enzymes is characterized by limited efficacy and significant side effects in some patients. Glucocorticoid steroids have antiinflammatory properties but may lead to a number of serious side effects when used acutely or chronically. More recently, anti-TNF agents have come to be used for the treatment of rheumatoid arthritis, with significant improvement in many symptoms, including pain. Unfortunately, an increased risk of serious infections, allergic reactions, skin problems, and a host of other side effects can accompany the use of these agents.[57] By selectively targeting one key component of the complement system supporting inflammation while leaving active much of the infection-fighting capacity of the complement system, it may be possible to reduce pain with agents providing a more favorable side effect profile. Even with a limited spectrum of activity, the use of complement receptor antagonists resulting in the reduction of cytokine formation in surgical wounds might have adverse effects on healing. For example, mice lacking expression of IL-6 display delayed wound healing.[58] The application of TNF-α to wounds has been described to have both beneficial and detrimental effects on wound healing.[59,60] Reviews are available describing the roles cytokines and other soluble mediators play in wound repair.[61] The authors suggest that future studies involving agents modifying cytokine production and pain sensitivity evaluate (1) effects on nociception, (2) effects on immunologic status, and (3) effects on wound healing. In this way, the principal issues surrounding the use of such potential analgesic agents may be addressed.

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