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Distinct Temporal Patterns of Macrophage-Inflammatory Protein-2 and KC Chemokine Gene Expression in Surgical Injury[1]

Brian Endlich,[†] David Armstrong,[*] Jason Brodsky,[†] Michael Novotny,[†] and Thomas A. Hamilton[2*]

In the present study the regulation of CXC chemokine expression was evaluated in full-thickness abdominal wounds in mice. During the first 24 h after injury, IL-1$\alpha\beta$, KC, macrophage-inflammatory protein (MIP)-2, and monocyte chemoattractant protein-1 were the predominant cytokines and chemokines produced; TNF-α was not detected. Chemokine mRNA expression and protein secretion occurred in two temporal stages. The first, which reached a maximum at 6 h, was associated with high levels of IL-1α and KC and low levels of MIP-2. This stage could be reproduced by intradermal injection of IL-1α or IL-1β and was partially blocked by injection of neutralizing Ab against IL-1α but not IL-1β. In animals depleted of circulating neutrophils, chemokine expression was reduced by nearly 70% during this stage. In the second stage, which peaked at 24 h after injury, modest but significant levels of IL-1β were detected in association with low levels of KC and high levels of MIP-2. This pattern of chemokine expression could not be mimicked by injection of IL-1α or IL-1β (even with prolonged exposure), although MIP-2 expression could be partially inhibited by intradermal injection of neutralizing Ab against IL-1β. Surprisingly, neutrophil depletion before injury resulted in sustained high levels of both KC and MIP-2 expression. These observations demonstrate that these two closely related chemokines are under distinct regulatory controls in vivo that are likely to reflect the temporally ordered participation of different cell types and/or extracellular stimuli and inhibitors. ***The Journal of Immunology,*** **2002, 168: 3586–3594.**

Surgical injury sets in motion a series of events which are, in normal physiology, essential for protection against microbial infection and for the restoration of preinjury tissue structure and function (1, 2). Immediate events involve cells that are resident in the tissue, but within minutes to hours of injury, mobile leukocytes, including granulocytes and monocytes, infiltrate the site and orchestrate much of the subsequent response (3, 4). While this process is highly complex and involves many different cell types and molecules, much recent attention has focused upon the production and secretion of chemokines, which regulate the trafficking of leukocytes and modulate their activities (5–9).

The chemoattractant cytokine or chemokine gene superfamily contains >40 members that can be subdivided into at least four families based upon the position of the first two cysteine residues in the amino acid sequence (6–8). The CC and CXC families include the majority of chemokines. Within the CXC family, there are at least two subgroups that are distinguished by the presence of a 3-aa sequence motif (ELR) that immediately precedes the CXC motif. There are at least four members of the ELR CXC chemokine family in the mouse, two of which (KC and macrophage-inflammatory protein (MIP)[3]-2), are believed to participate in neutrophil recruitment to sites of inflammation in many tissue locations (10–12). CXC chemokines are among the earliest new proteins produced at sites of inflammation, consistent with their role in regulating the early infiltration of neutrophils (13, 14). In contrast to the CXC family, the CC chemokine family exhibits broader target cell specificity including lymphocytes, eosinophils, basophils, and monocyte/macrophages (6–8).

The regulation of chemokine gene expression in vivo may be important in two respects. Certainly, the expression of gene products with inflammatory activities must be closely controlled to minimize unnecessary tissue damage. In addition, many chemokines exhibit functional redundancy but are expressed in distinct temporal and/or spatial patterns indicating differential regulation (6–8, 14). Furthermore, unlike many proinflammatory cytokines whose abundant expression is largely restricted to inflammatory leukocytes, almost all cells exhibit the potential to produce substantial amounts of proinflammatory chemokines in response to a broad spectrum of stimuli (6, 7). The prototypic proinflammatory cytokines IL-1$\alpha\beta$ and TNF-α are well recognized as among the most potent inducers of chemokine expression both in vitro and in vivo and have been demonstrated in many inflammatory sites within minutes to hours following injury (1, 2). Following their synthesis and/or release, these agents could stimulate chemokine expression in multiple surrounding cell types. The observed temporal patterns of chemokine expression may then reflect differential participation of distinct cell types and stimuli.

In the present study we examined the temporal expression patterns of the two major neutrophil-directed chemokines in the mouse (MIP-2 and KC) following an incisional skin wound. The

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[3] Abbreviations used in this paper: MIP, macrophage-inflammatory protein; MCP, monocyte chemoattractant protein; rm, recombinant mouse.

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results define two distinct stages during the first 24 h after injury; KC expression predominates during the first 12 h and declines by 24 h while MIP-2 expression is modest during the first phase, with highest expression at the 24-h time point. The two stages of chemokine expression depend in part on the release and de novo synthesis of IL-1α and IL-1β, respectively. Furthermore, the differential patterns of expression are dependent upon and/or regulated by the infiltration of inflammatory neutrophils in opposing fashion.

Materials and Methods

Reagents

Cesium chloride, guanidine thiocyanate, agarose, SDS, Tris, proteinase K, RNase-free DNase, and random priming kits were purchased from Boehringer Mannheim (Indianapolis, IN). Formamide was obtained from U.S. Biochemical (Cleveland, OH). Dextran sulfate was obtained from Pharmacia Biotech (Uppsala, Sweden). Nylon transfer membrane was purchased from Micron Separations (Westborough, MA). DuPont NEN Research Products (Boston, MA) was the source of [α-^{32}P]dCTP and Renaissance ECL reagent. ELISA kits for mouse cytokines and chemokines including TNF-α, IL-1α, IL-1β, KC, MIP-2, and monocyte chemoattractant protein (MCP)-1(JE), neutralizing polyclonal Abs against mouse IL-1α and IL-1β, and rIL-1α and rIL-1β were obtained from R&D Systems (Minneapolis, MN). A total of 0.3 ng of anti-IL-1α and 1 ng of anti-IL-1β Ab could neutralize as much as 25 pg of IL-1α or IL-1β, respectively. Rat anti-neutrophil mAb (no. MCA771G) was obtained from Serotec (Raleigh, NC) and the RB6-C85 mAb (15) was purified by protein G-Sepharose chromatography from cell culture supernatants. Biotinylated anti-rat Ig was obtained from Vector Laboratories (Burlingame, CA) and HRP-conjugated goat anti-rat IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Surgical wound

Six- to 8-wk-old female C57BL/6 mice (Charles River Breeding Laboratories, Wilmington, MA) were anesthetized with i.p. injection of sodium pentobarbital (75 mg/kg) and subjected to 20-mm full thickness abdominal incisions. The incisions were closed with Ethibond 5/0 braided nylon suture (Ethicon, Somerville, NJ). At specific times after wounding, mice were sacrificed by CO_2 asphyxiation and the tissue surrounding the wound site (4-mm border on both sides) was harvested and used for preparation of total RNA or total protein extract, or fixed for immunohistology. Each experimental group contained 6–10 animals.

RNA preparation, Northern hybridization, and RNase protection assays

Mouse tissue was homogenized for total RNA extraction by the guanidinium isothiocyanate-CsCl method as described previously (16, 17). The levels of cytokine and chemokine mRNA were determined either by Northern hybridization using radiolabeled cDNA fragments as described previously (16, 17) or by use of the RiboQuant RPA system (BD PharMingen, San Diego, CA) according to the manufacturer's instructions. Plasmids containing KC, MIP-2, and GAPDH cDNAs were prepared as described previously (18, 19).

Preparation of tissue extracts and ELISA

Tissue sections for measurement of cytokine protein levels were homogenized by mechanical disruption with a PowerGen tissue homogenizer (Fisher Scientific, Pittsburgh, PA) in 150 mM NaCl and 50 mm Tris-HCl

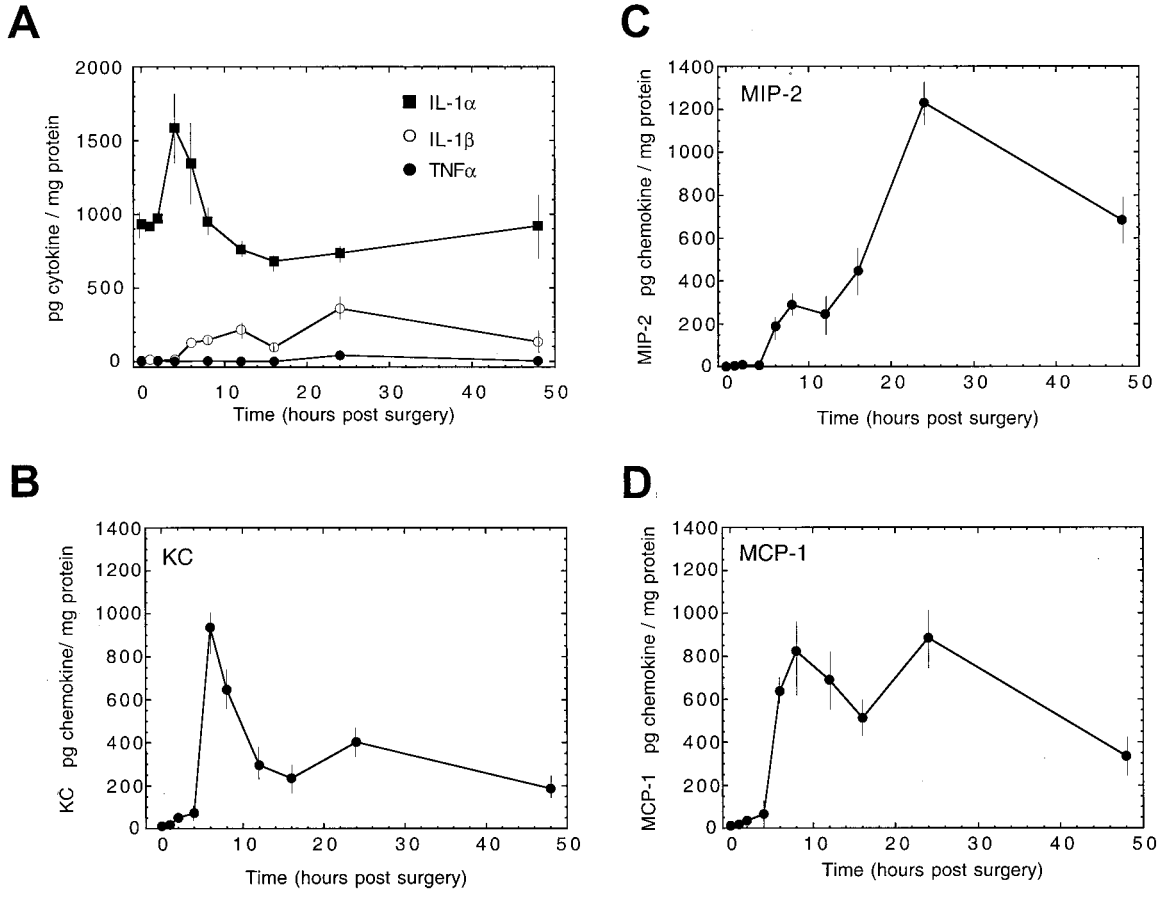

FIGURE 1. Time course of cytokine and chemokine expression in surgical wound tissue. C57BL/6 mice were anesthetized and subjected to a 20-mm full thickness abdominal skin incision and the wound was closed. At the indicated times, the animals were sacrificed, 8 × 6-mm sections of the wound site were excised, and tissue protein extracts were prepared as described in *Materials and Methods*. Aliquots were used to measure the indicated cytokines and chemokines by ELISA and normalized for protein content. *A*, IL-1α, IL-1β, and TNF-α. *B*, CXC chemokine KC. *C*, CXC chemokine MIP-2. *D*, CC chemokine MCP-1. Each data point represents the mean ± SEM for six animals.

(pH 7.4) with Minimix protease inhibitors (Roche Pharmaceuticals, Indianapolis, IN). Tissue homogenates were centrifuged at 18,000 × *g* for 10 min and the supernatants were collected, aliquoted, and stored at −20°C. Protein concentrations were measured by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Quantification of IL-1α, IL-1β, KC, MIP-2, MCP-1, MIP-1α, RANTES, and eotaxin was done using Quantitative Colorimetric Sandwich ELISA according to the manufacturer's instructions.

Immunohistochemistry

Tissue specimens were fixed in 10% buffered formalin for 18 h, dehydrated, embedded in paraffin, and cut in 5-μm sections. The slides were stained for neutrophils using a rat IgG anti-mouse neutrophil mAb (MCA771G). The secondary biotinylated anti-rat Ab was diluted 1/100 in PBS for use and subsequently followed by incubation with avidin-biotin peroxidase. Color development was performed with 3′,3′-diaminobenzidine and the slides were counterstained with hematoxylin.

Western slot-blot analysis

One microgram of each tissue extract was applied to nitrobind/nitrocellulose membrane (Osmonics, Westborough, MA) in a Bio-Dot apparatus (Bio-Rad) and subsequently rinsed with PBS. The slot-blot membrane was preincubated 1 h at 20°C in 5% nonfat dry milk in TBS plus Tween 20 (TBST). Rat anti-mouse neutrophil Ab (MCA771G) was used at a dilution of 1/5000 and incubated with filters for 1 h at 20°C. Primary Ab was removed, filters were washed, and secondary Ab (HRP-conjugated goat anti-rat IgG diluted 1/3000 in 5% milk/TBST) was added for an additional 1-h incubation at 20°C. Blots were developed using ECL reagent and quantified by densitometry using IMAGE software (National Institutes of Health, Bethesda, MD). The specificity of the anti-neutrophil Ab was assessed by analysis of cross-reactivity between elicited peritoneal neutrophils and macrophages obtained 6 and 48 h, respectively, after i.p injection of 1 ml of 3% Brewer's thioglycolate broth.

Intradermal injection

Recombinant cytokines or neutralizing polyclonal Abs were injected intradermally at three separate sites along a 20-mm segment of the abdominal midline using a 26-gauge needle and a tuberculin syringe. Cytokines and Abs were injected in a total volume of 100 μl. For animals undergoing surgery, injections were made 30 min before the procedure. For Ab neutralization studies, control mice were injected with comparable levels of nonimmune goat IgG. Tissue samples were harvested and used for analysis of cytokine and chemokine protein expression as described above.

Statistical analysis

Comparisons of treatment arms over the course of the study were performed with ANOVA F tests. If significant differences were found, then pairwise Student's *t* tests were used to compare groups. Standard errors were calculated using least-squares means techniques based on the two-way ANOVA. All calculations were performed with SAS version 8.1 software (SAS Institute, Cary, NC).

Results

Time course of the cytokine/chemokine cascade in surgical wound tissue

Injury induced by an incisional wound resulted in the production of multiple cytokines and chemokines within the first 24 h as measured by ELISA of tissue homogenates (Fig. 1). The most prevalent cytokines detected were IL-1α and IL-1β; no TNF-α, IL-6, IFN-γ, or TGFβ was observed in the initial 24 h following injury. The most highly expressed chemokines were KC, MIP-2, and MCP-1, while little or no MIP-1α, MIP-1β, eotaxin, or RANTES protein was measurable during this early time period. IL-1α was present in the tissue before injury, most likely sequestered within keratinocytes (20, 21). In addition, the levels of IL-1α increased rapidly to a peak at 4 h postsurgery, presumably due to de novo synthesis of IL-1α mRNA (data not shown). IL-1α protein declined to preinjury levels within 12 h and remained at this level throughout the experimental period. IL-1β mRNA and protein production commenced soon after wounding, but the amounts produced were markedly lower than IL-1α. This response was biphasic, reaching an initial peak around 4–8 h, followed by a modestly higher peak at the 24-h time point (Fig. 1*A*). Expression of the CXC chemokine KC exhibited a biphasic pattern in which early levels of KC production generally exceeded levels observed at the late peak (Fig. 1*B*). In contrast, though MIP-2 expression was also biphasic, the later component of the response was three to four times greater than the early component (Fig. 1*C*). Thus KC levels exceeded MIP-2 during the early phase while MIP-2 levels predominated during the later phase. The CC chemokine MCP-1 exhibited roughly equivalent expression at 8 and 24 h (Fig. 1*D*).

This differential pattern of chemokine expression was also demonstrable at the level of mRNA. Wound tissue was used to prepare total RNA from individual mice at various times after surgery, and this was analyzed by Northern hybridization to determine the level of specific chemokine mRNA expression. mRNA levels for KC were detected within 2 h of injury, reached a maximum between 4 and 8 h, and declined to resting levels between 16 and 24 h (Fig. 2). In contrast, MIP-2 mRNA was first detected at 4 h after injury and increased to maximal levels between 12 and 16 h after injury. These data demonstrate that the distinct pattern of KC and MIP-2 protein secretion results from alterations at the level of gene expression and not from differences in use or sequestration of secreted chemokine protein.

IL-1$\alpha\beta$ differentially regulate the temporal expression of KC and MIP-2

The presence of IL-1α and IL-1β in wound tissue in the absence of TNF-α suggested that IL-1 may be an important stimulus of CXC chemokine expression during the response to injury. As a first test of this possibility, mice were treated intradermally with neutralizing Abs against IL-1α or IL-1β 30 min before surgery.

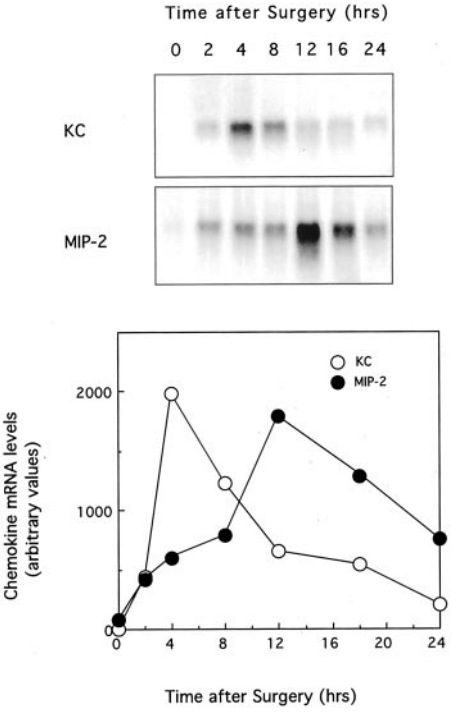

FIGURE 2. Time course of KC and MIP-2 mRNA expression. Sections of wound tissue obtained from individual animals at the indicated times following surgery were used to prepare total RNA for analysis of expression of KC and MIP-2 mRNA by Northern hybridization as described in *Materials and Methods*. The autoradiograph was quantified by image analysis using the National Institutes of Health IMAGE software. Similar results were obtained in two separate experiments involving multiple animals at each time point.

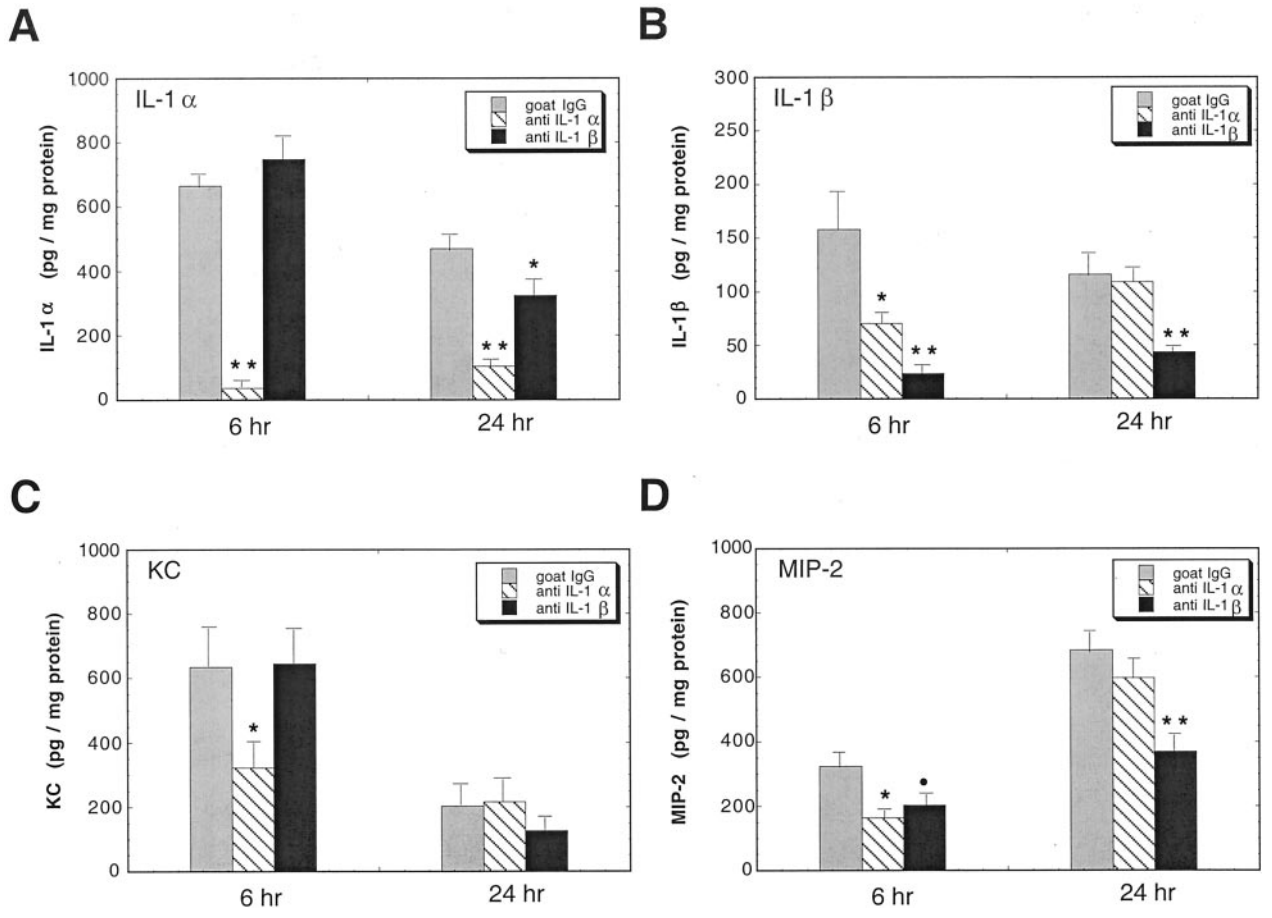

FIGURE 3. Distinct temporal roles for IL-1α and IL-1β in chemokine expression following surgery. C57BL/6 mice were injected intradermally with 50 μg of goat Ig (nonimmune or specific for IL-1α or IL-1β) in 100 μl of saline 30 min before surgery. Injections were given in 30-μl increments placed at three positions along the 20-mm abdominal incision line. Animals were sacrificed at the indicated times, and tissue sections were obtained and used to prepare protein extracts as described in Fig. 1. Extracts were used to measure the indicated cytokines or chemokines by ELISA. *A*, IL-1α (*, $p = 0.001$; **, $p < 0.0001$). *B*, IL-1β (*, $p = 0.02$; **, $p < 0.0001$). *C*, KC ($p = 0.02$). *D*, MIP-2 (●, $p = 0.03$; *, $p = 0.008$; **, $p < 0.0001$). Each data point represents the mean ± SEM for six animals.

Injection of 50 μg of anti-IL-1α or anti-IL-1β reduced the detectable levels of the cognate cytokine by >95% (Fig. 3, *A* and *B*). Though these Abs exhibit <1% cross-reactivity, neutralizing IL-1α also reduced the level of IL-1β measured 6 h following surgery. Neutralizing IL-1α with 50 μg of Ab blocked the production of KC and MIP-2 by ~50% at the 6-h time point, while neutralization of IL-1β reduced MIP-2 levels but had no effect on KC levels (Fig. 3, *C* and *D*). Consistent with the finding that TNF-α was not expressed, no effect was observed following injection of neutralizing Ab directed against this cytokine (data not shown). When chemokine expression was measured 24 h following Ab injection and surgery, neutralization of IL-1α had little effect on either chemokine, whereas neutralization of IL-1β caused a marked reduction in expression of both KC and MIP-2 (Fig. 3, *C* and *D*). The neutralizing efficacy of the Ab treatment at 24 h after injury may be diminished due to the diffusion of Ab from the tissues. Nevertheless, these findings indicate that IL-1α and IL-1β contribute differentially to the temporally distinct waves of KC and MIP-2 expression.

The role of IL-1α in the induction of IL-1β, KC, and MIP-2 was further tested by injection of 100 ng of recombinant mouse (rm)IL-1α intradermally at three different sites along a 20-mm section of the abdomen. Injection of saline vehicle caused no change in cytokine or chemokine expression. Injection of rmIL-1α had a modest effect on the expression of IL-1β but did not induce TNF-α (Fig. 4*A*). KC and MIP-2 were both strongly induced by IL-1α, exhibiting a sharp peak at 4 h which declined to near basal levels by 12 h postinjection (Fig. 4*B*). Both chemokines were expressed at levels higher than observed following surgery, but the quantitative relationship between the two was similar to that seen during the early postsurgical phase (e.g., KC ≥ MIP-2). Notably, no secondary wave of chemokine expression was observed. KC expression was also more sensitive than MIP-2 to IL-1α, as indicated by an ~5-fold difference in the threshold dose required to elicit expression of either chemokine (Fig. 4*C*). Both IL-1α and IL-1β induced comparable patterns of expression following a single injection (Fig. 4*D*). Hence the differential roles for IL-1α and IL-1β depend upon their temporal expression patterns rather than their inherent capacity to stimulate chemokine expression. The lack of prolonged chemokine expression seen with a single bolus injection of IL-1 might reflect a requirement for continued exposure to the stimulus. Indeed, in mice injected three times at 8-h intervals, KC expression remained high. However, even under these conditions MIP-2 expression was only modest (Fig. 4*D*). These findings demonstrate that IL-1 functions as a relatively selective stimulus for KC expression in the skin.

Role of IL-1 in neutrophil infiltration

To test the role of IL-1$\alpha\beta$ in neutrophil recruitment to the wound site, tissue from operated animals or animals injected with

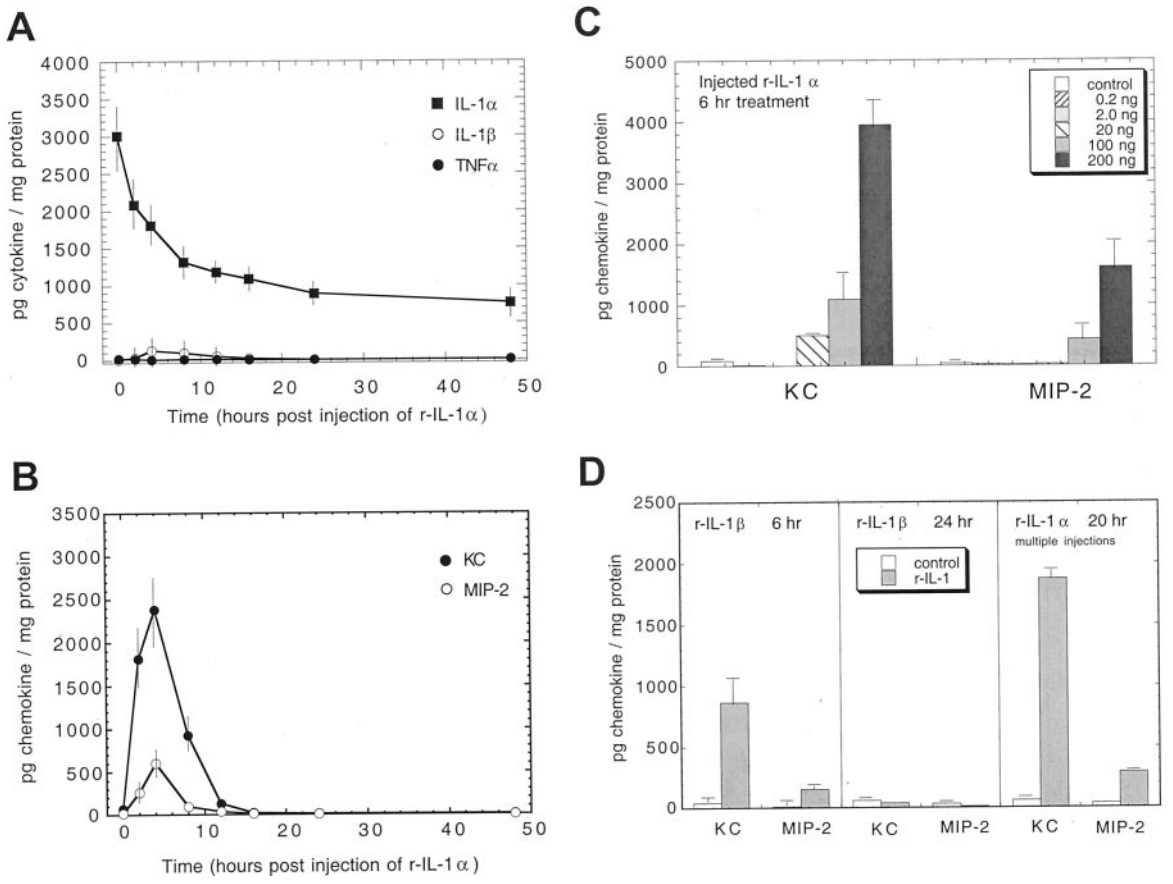

FIGURE 4. Injection of rmIL-1 promotes cytokine and chemokine expression in the skin. A total of 100 ng or the indicated amounts of rmIL-1α or rmIL-1β was injected intradermally at three sites along a 20-mm midline section of the abdomen. At the indicated times, the animals were sacrificed and 6 $\times$ 8-mm sections were excised and used to prepare tissue protein extracts. Cytokine and chemokine protein levels in each sample were quantified by ELISA and normalized to protein concentration. *A*, rmIL-1α-induced expression of IL-1α, IL-1β, and TNF-α. *B*, rmIL-1a-induced expression of KC and MIP-2. *C*, Dose dependence for rmIL-1α-induced KC and MIP-2 expression measured at 6 h postinjection. *D*, rmIL-1β- or rmIL-1a (100 ng each)-induced expression of KC and MIP-2 at 6 and 24 h postinjection (panels 1 and 2) or at 20 h following three injections given at 8-h intervals (t = 0, 8, 16 h) (panel 3). Each data point represents the mean $\pm$ SEM of six animals.

rmIL-1α was harvested at 6 or 24 h postinjury or postinjection for analysis of neutrophil content. The magnitude of neutrophil infiltration was assessed by measuring the amount of neutrophil-specific Ag present in tissue extracts using a Western immunoblot strategy, while the distribution of infiltrating neutrophils was assessed by immunohistology. One-microgram aliquots of tissue extract were blotted onto filters using a slot-blot apparatus and probed with a neutrophil-specific mAb. This method was relatively specific for neutrophils as indicated by a lack of reactivity with extracts from untreated skin. Furthermore, there was <3% cross-reactivity using extracts obtained from purified populations of peritoneal neutrophils and macrophages. A time course of representative samples from individual operated mice over a period of hours to days illustrates that neutrophils are first detected at the wound site within 4 h, reach maximum levels by 24 h, and disappear gradually over the next 2–3 days (Fig. 5). This finding is confirmed by the immunohistology results, which illustrate that the neutrophil infiltrate is much greater at 18–24 h after surgery than at 6 h and that the primary site of infiltration is at the wound edge (Fig. 6, *A–C*). When neutrophil infiltration was evaluated in tissues from animals injected with rmIL-1α or β, the response was quantitatively less than that observed in operated animals. While the leukocyte content in operated and IL-1α-injected mice was comparable through the first 12 h, it increased through 48 h in operated animals but declined in injected animals between 12 and 48 h (Figs. 5*B* and 6, *D–E*). The distribution of neutrophils in IL-1 injection sites was diffuse compared with the pattern seen in operated animals. The injection of saline vehicle did not produce detectable leukocytic infiltration.

Neutrophils regulate chemokine expression

Neutrophils have been shown to be important sources for chemokine expression (13, 14, 22–24). In addition, both chemokine expression and neutrophil infiltration differed dramatically between operated and IL-1-injected animals. Hence we wished to assess the importance of neutrophils in expression of KC and MIP-2 at sites of surgical injury. Mice were depleted of circulating neutrophils by i.p. injection of 100 μg of the anti-neutrophil mAb (RB6-C85) as previously described (15, 25). This treatment led to a 95% reduction in the number of neutrophils elicited into the peritoneal cavity 6 h following i.p. injection with thioglycolate broth. One day after Ab treatment animals were either operated or injected intradermally with rmIL-1α (single or multiple injections) and the wound or injection sites were harvested 6, 24, or 48 h later. Examination of wound or injection sites immunohistochemically or by slot-blot analysis showed that neutrophil infiltration was undetectable at 6 h and reduced to <20% of control values at 24 h after surgery (data not shown). This reduction in leukocyte infiltration results in a marked diminution in expression of both KC and MIP-2 at the wound site during the early phase of the response (6 h) (Fig. 7).

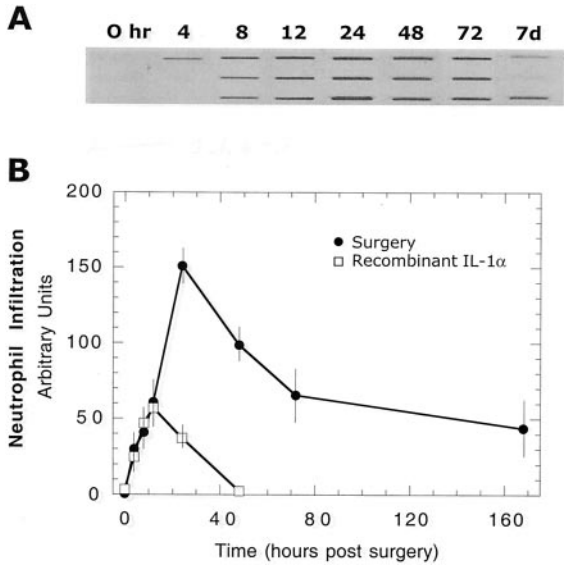

FIGURE 5. Neutrophil infiltration in operated or IL-1-injected skin. Tissue samples from the animals used for experiments described in Figs. 1 and 4 were used to quantify the magnitude of neutrophil infiltration using a Western slot-blot methodology. Samples of each protein extract (1 μg) were applied to nitrocellulose filters and developed with anti-neutrophil Ab as described in *Materials and Methods*. Blots were developed using ECL and the film exposures were quantified by image analysis using the National Institutes of Health IMAGE software. Results represent arbitrary values corresponding to optical densities of individual bands. *A*, ECL exposure of Western slot-blot displaying representative tissue samples from operated mice obtained over a 7-day time course following surgery. *B*, Samples from operated or rmIL-1α (100 ng)-injected mice shown in Figs. 1 and 4, respectively, were processed as in *A* and films were quantified by image analysis. Each data point represents the mean ± SEM of seven animals.

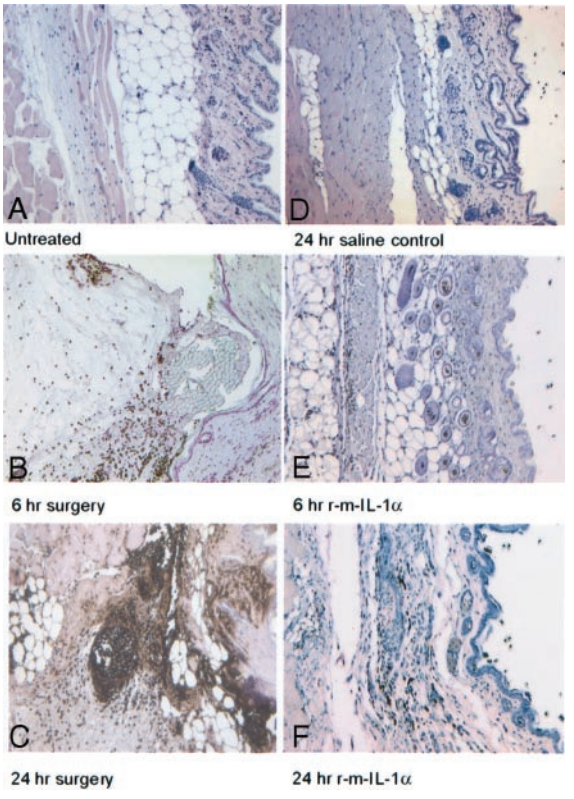

FIGURE 6. Immunohistochemical analysis of neutrophil infiltration. Sections of skin (6 × 8 mm) from selected experiments described in Figs. 1 and 4 were fixed in Histochoice (Sigma-Aldrich, St. Louis, MO) and processed for immunohistochemistry using an anti-neutrophil mAb as described in *Materials and Methods*. *A*, Normal skin tissue. *B*, Wound site 6 h postsurgery. *C*, Wound site 24 h postsurgery. *D*, Skin injected with normal saline. *E*, Skin 6 h postinjection of 100 ng rmIL-1α. *F*, Skin harvested 24 h postinjection of 100 ng of rmIL-1α. *B* and *C*, The wound margin runs through the center of the image from *top* to *bottom*.

Surprisingly, by 24 h, levels of both KC and MIP-2 were markedly higher than those seen in neutrophil-replete animals. By 48 h after injury, KC levels returned to baseline in both normal and neutrophil-depleted animals, while MIP-2 levels remained elevated in both cases. The effects of neutrophil depletion in operated animals were most dramatic on KC expression; in normal animals KC levels declined substantially by 24 h, while in neutrophil-depleted animals KC levels at 24 h markedly exceeded even those seen in normal animals at the peak of expression. In neutrophil-depleted animals injected intradermally with rmIL-1α, KC and MIP-2 levels were comparable or modestly reduced as compared with controls at both 6 and 20 h (in animals receiving three consecutive IL-1α injections). As in prior studies (see Fig. 4), IL-1 injection was a potent stimulus of KC expression but had only a modest effect on MIP-2 (Fig. 7).

Discussion

The early phase of inflammatory response following traumatic injury includes the activation of a cytokine-chemokine cascade associated with the infiltration of inflammatory leukocytes (1, 2, 4). The process of leukocyte infiltration depends, in part, on the action of chemoattractants, specifically members of the chemokine gene family (5–9). The potential tissue damage associated with recruitment of inflammatory leukocytes demands that expression of these genes be subject to stringent control. Furthermore, previous studies of chemokine expression in various inflammatory settings in vivo have demonstrated distinct temporal and/or spatial patterns of expression for functionally similar chemokine genes (14, 26–28). Hence it is necessary to understand how chemokine expression is controlled in vivo. The objective of the present study was to determine patterns of neutrophil-directed CXC chemokine expression in the skin of mice following surgical injury and to identify regulatory controls involved. The results demonstrate that the cytokine-chemokine cascade invoked following surgical wounding is biphasic and that functionally comparable chemokines KC and MIP-2 exhibit very different temporal patterns of expression that result from distinct regulatory controls operating in cell type- and stimulus-dependent fashion. These conclusions are supported by the following experimental findings: 1) early cytokine (IL-1$\alpha\beta$) and chemokine (KC, MIP-2, and MCP-1) expression occurs in two distinct temporal waves; 2) KC and MIP-2 are expressed differentially, as KC exceeds MIP-2 early (6 h after injury) while MIP-2 predominates during the later phase (24 h); 3) IL-1 is a potent stimulus of KC expression but has only modest activity for MIP-2 (even prolonged exposure to IL-1 cannot reproduce the second, MIP-2-dominated stage of chemokine expression); and 4) infiltrating neutrophils influence the two stages in opposing fashion; in neutrophil depleted animals, chemokine expression during the first stage is reduced while KC expression during the second stage is markedly enhanced.

The temporal sequence of cytokine and chemokine expression at a wound site during the first 24 h appears to be at least biphasic, and the major components (IL-1$\alpha\beta$, KC, MIP-2, and MCP-1) each exhibited an early and late response pattern. Complex temporal

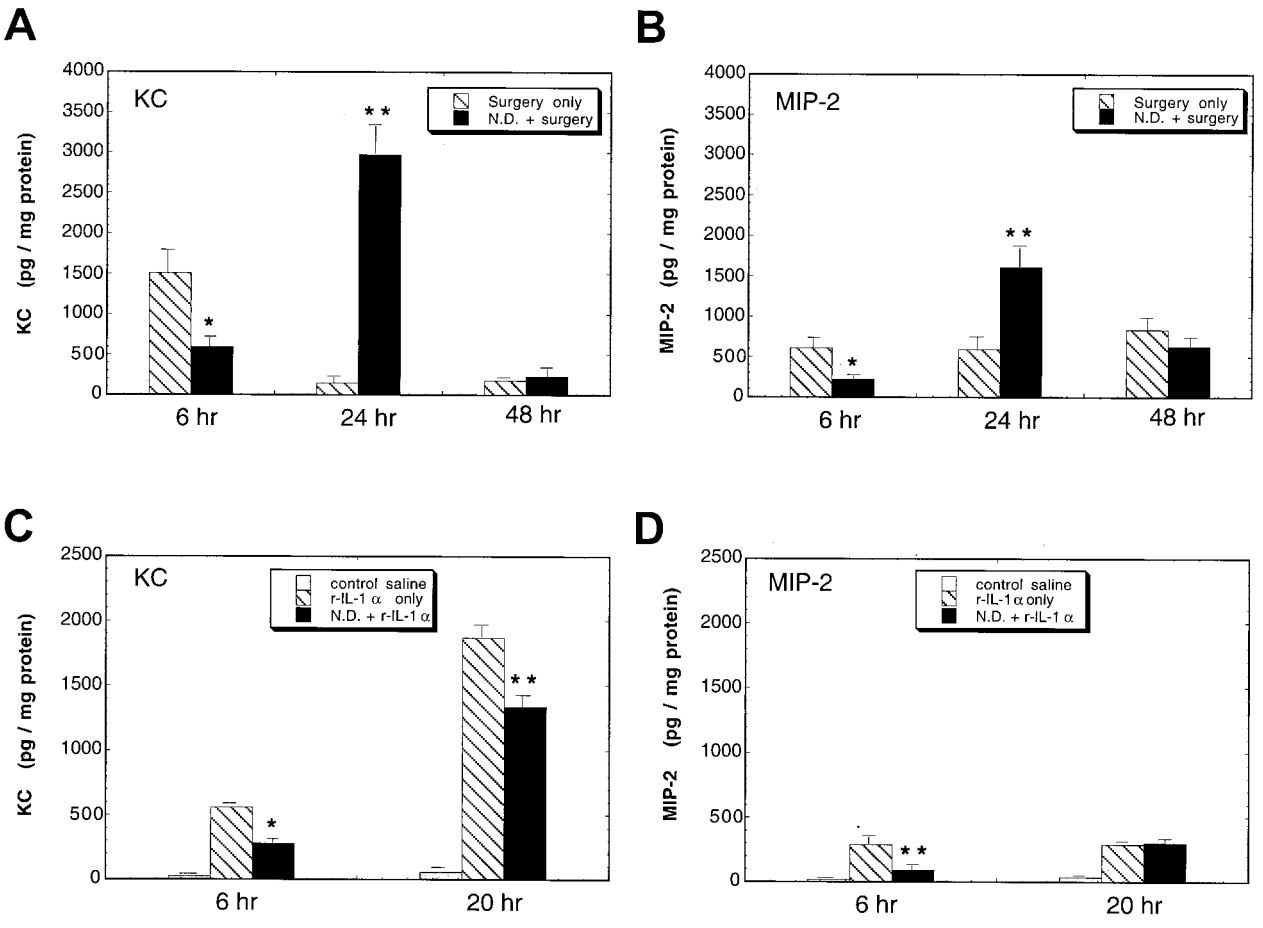

FIGURE 7. Effects of neutrophil depletion on surgery and IL-1-stimulated chemokine expression. C57BL/6 mice were injected i.p. with vehicle or 100 μg of anti-neutrophil mAb (ND, neutrophil depleted) 24 h before surgery or intradermal injection of rmIL-1a (as described in Fig. 4). At 6 or 24 h after surgery or injection, the animals were sacrificed and tissue samples were used to prepare protein extracts for determination of chemokine levels by ELISA. *A*, KC, surgery (*, $p < 0.005$; **, $p < 0.0001$). *B*, MIP-2, surgery (*, $p < 0.05$; **, $p < 0.0001$). *C*, KC, rIL-1α (*, $p = 0.01$; **, $p < 0.0001$). *D*, MIP-2, rmIL-1α (**, $p < 0.0001$). Each data point represents the mean ± SEM of six animals.

patterns of chemokine expression in vivo have been previously reported in a number of settings, although the regulation of such expression has not been explored in detail (13, 14, 26–30). The distinct patterns of expression for the different CXC chemokine genes reported in this study are consistent with studies of chemokine expression in excisional wounds in human skin measured between 1 and 7 days after injury (13, 14). However, the present study focuses upon patterns observed within the first 24 h after injury. IL-1α and β appeared to be the predominant cytokines seen within minutes to hours after injury. IL-1α was detected in uninjured skin, most likely in preformed pools within keratinocytes (20, 21), and was released into the tissue rapidly after surgery. In contrast, IL-1β protein secretion was induced at modest levels but exhibited two distinct temporal phases with peaks at ~4–6 h and at 24 h following injury. Although these two isoforms of IL-1 appear to have comparable stimulatory activity for promoting chemokine expression in the skin, the use of neutralizing Abs suggests that they operate in a temporally distinct fashion; IL-1α is present and active within the first phase of response (up to 10 h after injury) while IL-1β contributes stimulatory activity in the second phase of response. The very low induction of TNF-α observed in this model is surprising and in some conflict with previous literature (29, 31). The differences in our results from those previously reported may reflect differences in the methodologies used (RT-PCR vs ELISA) or use of different mouse strains and/or wound models.

The patterns of KC and MIP-2 expression were markedly different from one another; while KC expression was substantially higher than MIP-2 during the first phase of response, MIP-2 levels significantly exceeded KC levels in the second phase of the response. A number of prior studies have observed differential expression both in terms of cell types and in terms of temporal pattern (32–37). For example, in several models of bacterial infection in mouse lung, maximum KC expression occurs at day 1 while MIP-2 peaks on day 2 (34, 36). In models of ischemia/reperfusion injury and during peritoneal inflammation resulting from cecal ligation/puncture, the temporal pattern of KC and MIP-2 expression differs and appears to involve distinct cell populations (33, 35). The majority of these studies have studied expression at 24 h and later; whether the regulatory mechanisms are similar to those operative during the first 24 h following injury is not known. Interestingly, the differences between KC and MIP-2 expression in terms of time and magnitude suggest that these two functionally similar chemoattractants may have nonredundant roles in the inflammatory process. Indeed, because expression of MIP-2 reaches peak levels at or after the peak of neutrophil infiltration has occurred, MIP-2 may function in later aspects of the inflammatory response. In this regard, CXC chemokines have been reported to exhibit angiogenic activity (38, 39), and several studies have suggested important roles for CXC chemokines and CXCR2 in wound healing. Such an activity would be consistent with the pattern of MIP-2 expression observed in this study (40, 41).

The markedly different expression kinetics for KC and MIP-2 demonstrated in this work are most consistent with phase-specific regulation of each gene by distinct stimuli and/or cell types. Injection of rmIL-1α or rmIL-1β appears to reproduce the early pattern of chemokine expression in which KC predominates over MIP-2. Both IL-1α and IL-1β were capable of stimulating this early response, but neither agent was able to stimulate a pattern of response in which MIP-2 predominates even when tissues levels of IL-1 were maintained over the 24-h time frame by multiple injections. This suggests that the early wave of chemokine expression (in which KC predominates) is dependent upon cells resident in the injured tissue while the second phase may require additional cell types and/or stimuli that are only found in operated animals.

The observation that neutrophil infiltration was modest in IL-1-injected as compared with operated animals suggested the possibility that neutrophil infiltration might be an important regulatory aspect of chemokine gene expression. Indeed, neutrophils exhibit substantial chemokine gene expression in vitro and have been shown to be an important source of chemokine production in vivo (13, 14, 22–24). The depletion of neutrophils had very dramatic but surprising impact on the pattern of chemokine expression. The marked reduction of chemokine levels at 6 h following surgery in neutrophil-depleted animals indicates that at least a portion of the early burst of KC production is derived from or dependent upon infiltrating neutrophils. The truly remarkable increase in KC expression at 24 h after surgery in neutrophil-depleted animals, though unexpected, is consistent with a recent report of MIP-2 and KC expression in CXCR2$^{-/-}$ mice with intracranial infections (37). This observation suggests that KC expression does not decline naturally but is turned off as a consequence of the heavy neutrophil infiltrate. Though possible, this effect is not likely to result from the sequestration of secreted chemokine by neutrophils, because the diminution in KC expression is also seen at the mRNA level. Furthermore, stimuli inducing expression of both KC and MIP-2 must be present at the site during the period from 16 to 24 h following injury, because expression of both genes is very high in the absence of neutrophil infiltration. Hence, in normal animals, neutrophils at the site 16–24 h after injury may act to 1) prevent the generation of stimulus, 2) sequester the stimulus, or 3) inhibit the subsequent response. Moreover, this effect acts in partially selective fashion as KC expression is more sensitive than MIP-2. Neutrophil production of stimulus antagonists such as IL-1RA or soluble TNFR could sequester the necessary stimuli as suggested (42). Alternatively, because neutrophils likely undergo apoptosis at the wound site and apoptotic cell bodies have been shown to be anti-inflammatory, this process may provide a source of selective chemokine regulation (43, 44).

Animals injected multiple times with rmIL-1α exhibited prolonged high levels of KC expression with only modest neutrophil infiltration. Hence, the inflammatory cell infiltration that follows the initial surgical injury probably depends on chemoattractants other than KC and MIP-2. Indeed, a broad range of chemotactic stimuli may be generated at wound sites and provide partially redundant control of leukocyte infiltration (45). For example, the activation of complement and coagulation cascades associated with the traumatic wound are likely contributors to this early phase. The chemotactic activity of the C5a fragment of complement has long been recognized (46). Numerous components of the coagulation system have also been demonstrated to exhibit inflammatory properties (47). Furthermore, the tissue necrosis certain to be part of traumatic injury is believed to be a general stimulus of proinflammatory cytokine expression, although the molecular details of this effect are largely unknown (48, 49).

The findings presented in this study define, in detail, the patterns of cytokine and chemokine response which occur within the tissue microenvironment following surgical injury. The results illustrate a biphasic response that appears to be composed of distinct cell types and stimuli functioning in a temporally ordered fashion. The candidate cell types and stimuli that are likely participants have been identified both in vitro and in vivo in many previous studies. Furthermore, work with cell culture models has determined many of the molecular pathways that mediate response to external stimuli and translate such signals into altered patterns of gene expression. At present, however, the specific roles for individual cell types, extracellular stimuli, and intracellular regulatory mechanisms in producing the specific patterns of chemokine gene expression and their inflammatory sequellae in vivo remain to be fully appreciated.

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