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Identification of Unique Truncated KC/GROβ Chemokines with Potent Hematopoietic and Anti- Infective Activities

Andrew G. King,1* Kyung Johanson, † Carrie L. Frey, ‡ Peter L. DeMarsh, § John R. White, ** Patrick McDevitt, † Dean McNulty, † Joanna Balcarek, ‡ Zdenka L. Jonak, †† Pradip K. Bhatnagar, ††† and Louis M. Pelus* 

SK&F 107647, a previously described synthetic immunomodulatory peptide, indirectly stimulates bone marrow progenitor cells and phagocytic cells, and enhances host defense effector mechanisms in bacterial and fungal infection models in vivo. In vitro, SK&F 107647 induces the production of a soluble mediator that augments colony forming cell (CFU-GM) formation in the presence of CSFs. In this paper we purified and sequenced the stromal cell-derived hematopoietic synergistic factors (HSF) secreted from both murine and human cell lines stimulated with SK&F 107647. Murine HSF is an N-terminal 4-aa truncated form of the CXC chemokine, KC, while human HSF was identified as an N-terminal 4-aa truncated form of the CXC chemokine, GROβ. In comparison to their full-length forms, truncated KC and truncated GROβ were 10 million times more potent as synergistic growth stimulants for CFU-GM. Potency of these novel truncated chemokines relative to their full-length forms was also demonstrated in respiratory burst assays, CD11b Ag expression, and intracellular killing of the opportunistic pathogen, Candida albicans. Administration of truncated KC significantly enhanced survival of mice lethally infected with C. albicans. The results reported herein delineate the biological mechanism of action of SK&F 107647, which functions via the induction of unique specific truncated forms of the chemokines KC and GROβ. To our knowledge, this represents the first example where any form of KC or GROβ were purified from marrow stromal cells. Additionally, this is the first demonstration of in vivo efficacy of a CXC chemokine in an animal infectious fungal disease model. The Journal of Immunology, 2000, 164: 3774–3782.

Chemokines are potent chemoattractant proteins that display an ever-increasing spectrum of biological activity (1, 2). The chemokines discovered to date have been classified into four different families. These families are defined structurally by the invariant position of cysteine residues, C-C, C-X-C, C, and CX3C, in the N-terminal region of the protein. Chemokines are well-characterized ligands that attract host defense effector cells (neutrophils, monocyte, and lymphocytes) in vitro and in vivo along concentration gradients (1–3). Increasing evidence also suggests chemokines are important regulators of hematopoiesis (4). Hematopoietic chemokines with both enhancing and suppressive effects on bone marrow progenitors have been reported (5, 6). In this paper we describe the production and isolation of novel truncated forms of the human and mouse C-X-C chemokines GROβ and KC, respectively, which have potent hematopoietic enhancing activities and effector cell activation properties. Specific truncated chemokines were produced in response to stimulation by SK&F 107647 ((s)-5-oxo-I-prolyl-L-glutamyl-L-aspartyl-N2-(5-amino-I-carboxypentyl)-8-oxo-N2-[N-(5-oxo-I-prolyl)-L-glutamyl]-L-aspartyl]-2,7,8-triaminoocanoyl-L-lysine), 2 a new synthetic peptide related to the dimeric hematoregulatory peptide HP5B, first described by Laerum et al. (7). SK&F 107647 indirectly stimulates myeloid stem and progenitor cell proliferation, elevates host defense effector cell respiratory burst and antimicrobial activity in vivo, and enhances survival in animal models of infectious diseases (8–10). Mechanistically, the activities of SK&F 107647 are indirectly mediated by a soluble factor produced by marrow stromal cells which synergizes with all myeloid CSFs. We have identified, purified, and sequenced specific murine and human stromal cell-derived hematopoietic synergistic factors (HSF) induced by SK&F 107647, which mediate the in vivo effects of this peptide. Murine HSF is an N-terminal 4-aa truncated form of KC (aa 5–72), a member of the C-X-C chemokine family of proteins (11, 12). The murine KC gene is homologous to a superfamily of related human chemotactic heparin binding proteins, with high homology to the GROα, β, γ gene family (13). Both heparin and anti-GROβ mAb completely neutralize all SK&F 107647-induced HSF bioactivity in vitro and in vivo. Subsequently, we utilized heparin binding and anti-GROβ affinity chromatography to purify human HSF from a marrow stromal cell line, TF274. Human HSF was identified as an N-terminal truncated form of GROβ (5–73) by amino acid sequence and mass spectrometry. In this paper we use KC-T and GROβ-T to represent the specific 4-aa N-terminally truncated chemokines isolated from cell supernatants after SK&F 107647 stimulation; and KC and GROβ, to refer to the full-length forms of these proteins. Recombinant KC-T and GROβ-T proteins were cloned and produced, which confirmed the biological activities of the truncated chemokines produced in SK&F 107647-stimulated stromal cell cultures.

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Materials and Methods
Bone marrow stromal cell lines

The murine bone marrow stromal cell line, C6, was derived as previously described (14) and used as the source of SK&F 107647-induced synergistic activity. A transfected human stromal cell line, TF274, was utilized as the source of human SK&F 107647-induced synergistic activity. Briefly, the TF274 stromal cell line was derived from normal human bone marrow stimulated in vitro for 24 h with 0.003% Staph A, before transfection with HL-60 DNA and PzipTet (SV40 large T) used as an immortalizing agent, and delivered by electroporation. Clone TF274 was chosen based upon stromal cell morphology and responsiveness to SK&F 107647. Stromal cell lines were grown in either roller bottles (C6) or T-175 tissue culture flasks (TF274) and were treated with either 10 μg/ml (C6) or 100 μg/ml (TF274) SK&F 107647 under serum-free conditions. Cell-free supernatants were collected from treated cells after overnight stimulation. This process was repeated twice weekly. Cultures were washed extensively and refed with FCS-containing medium between treatment cycles.

CFU-GM assay

Murine bone marrow cells were harvested from 6- to 12-wk-old female C57BL/6 mice (Harlan Sprague-Dawley, Indianapolis, IN) and cultured as described (8). Cell supernatants from C6 or TF274 cells stimulated by SK&F 107647 (10–100 μg/ml) were collected, passed through 30,000 m.w. cut-off membranes (Centricron, Amicon, Beverly, MA), and tested for direct CFU-GM colony stimulating activity as well as for the presence of synergistic activity. Suboptimal concentrations of recombinant human M-CSF (Cellular Products, Buffalo, NY) were combined with dilutions of stromal cell supernatants, mouse serum, purification column fractions, or recombinant proteins and added to cultures of 7.5 × 10⁴ murine bone marrow cells in semisolid agar. CFU-GM colonies (>50 cells) were enumerated microscopically at 7 days postincubation at 37°C in 7.5% CO₂, 5% O₂ in humidified air.

Protein purification and characterization

Amino acid sequence analysis. N-terminal amino acid sequence analysis was performed on an Applied Biosystems (San Jose, CA) model 470A gas-phase protein sequencer equipped with a Beckman (Fullerton, CA) 126/166 system for on-line phenylthiohydantoin analysis. Data were acquired using System Gold chromatography software (Beckman, Fullerton, CA). Samples were spotted directly onto Polybrene coated GF/C filters (Applied Biosystems) and standard Applied Biosystems sequencing cycles used.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). MALDI-MS data on active fractions were obtained on an Applied Biosystems (WaveMetrics, Lake Oswego, OR) 100 laser scans. Calibrations were conducted using a customized version of IGOR Pro. Samples were prepared by mixing analyte 1:2 with 3,5-dimethoxy-4-hydroxy-cinnamic acid (Alphard, Milwaukee, WI), prepared as a saturated solution in 33% CH₃ CN/H₂O (v/v). Amino acid sequence analysis was performed on an Applied Biosystems (San Jose, CA) model 470A ear time-of-flight mass spectrometer. Samples were prepared for analysis with 1% trypsin in 50 mM Tris at pH 8.5 by air oxidation. Refolded proteins were purified by MALDI-MS analysis confirmed the homogeneity of the preparation of all chemokine isoforms constructed.

Preparation of polyclonal affinity purified anti-KC antiserum

New Zealand White rabbits were immunized with KC peptide aa 31–52 with CFA. KC-specific IgG was purified from the serum of boosted rabbits on an immobilized KC affinity column. Control rabbit IgG was purchased from Accurate Chemical Scientific (Westbury, NY).

Effector cell enrichment/functional analysis

Resident murine peritoneal macrophages. Female C57BL/6 mice were sacrificed by CO₂ asphyxiation. Following sacrifice, mice were injected with 10 ml of cold calcium and magnesium-free HBSS containing 10 U/ml heparin. The peritoneal cavity was gently massaged and the lavage fluid withdrawn and pooled. Peritoneal cell suspensions were adjusted to 4 × 10⁷/ml in RPMI 1640 with 1% heat-inactivated (HI-) FCS (HyClone, Logan, UT) and 50 μl added to microtiter wells and incubated for h at 37°C, 5% CO₂ to allow adherence. Following a 2-h adherence period, cells were removed by washing with warm HBSS and fresh RPMI 1640 with 10% FCS added to the cell monolayers (>95% macrophages as determined by microscopic morphology).

Human peripheral blood leukocytes. Peripheral blood was obtained from normal healthy volunteers by venipuncture, following informed consent. Suspensions of purified human neutrophils were prepared by dextran sedimentation followed by lysis of residual RBCs with 10 mM Tris/0.83% NH₄Cl buffer (pH 7.4) for 10 min. Mononuclear cells were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Cell suspensions were washed resuspended in HBSS, and cell counts and differentials were obtained using a Technicon H1 Hematology Analyzer (Miles Diagnostics, Tarrytown, NY). Viability was >97% as determined by trypan blue dye exclusion. Monocytes (>80%) were prepared by incubating mononuclear cells in RPMI 1640 with 1% HI-FCS in microtiter wells followed by the removal of nonadherent cells as described above.

Neutrophil chemotaxis. PMNs were washed twice with PBS and resuspended in PBS containing 1 mM MgCl₂ and 1 mM CaCl₂. Cell motility was determined using a modified Boyden chamber procedure as described (15). For measurement of chemotaxis, lower chambers were filled with 30 μl of IL-8, GRO₁, or GRO₂ (0.3–300 nM), the empty upper chambers were lowered into place and 50 μl of a PMN suspension (5 × 10⁶ cells/ml). PMN migration proceeded for 60 min at 37°C in the cell incubator after which the chamber was disassembled. Following fixation (75% methanol) and staining (Diff-Quick; Baxter, Miami, FL), migrated cells were counted in four successive high power fields.

Ca²⁺ mobilization in neutrophils. Human polynuclear leukocytes (PMNs) were separated from whole blood of healthy volunteers by the one-step Hypaque-Ficoll method (16). Cells were loaded with fura-2AM as described previously (17). Agonists were added at the indicated concentrations to 10⁶ cells/ml in Krebs-Ringer-Henseleit buffer. The maximal calcium concentration attained after agonist stimulation was quantitated as previously described (17).

Superoxide anion release. The superoxide dismutase (SOD)-inhibitable superoxide released by 2 × 10⁹ mouse peritoneal cells, human neutrophils, or human monocytes in response to in vitro stimulation by KC were measured in a microtiter plate assay. PMNs were washed and incubated in a microtiter plate at 37°C in a 5% CO₂ incubator. Following a 1-h incubation, 50 μl of PMN suspension (10⁶ cells/ml) was added to each well. The assay was performed in HBSS with 1% gelatin (Sigma), containing 80 μM cytochrome c (Sigma) in a final volume of 200
μl/well. The OD_{550} of each well was determined at 10-min intervals for up to 1-h incubation at 37°C and 5% CO₂. The amount of cytochrome c reduced/well was quantitated. Wells containing all of the reagents plus SOD (200 U/well; Sigma) were run concurrently to determine the maximal amount of SOD-inhibitable cytochrome c reduction. Control superoxide activity was determined in the presence of PBS alone. Data are expressed as a percentage of the control PBS response at peak response times.

**CD11b expression.** Blood cells from PBS or SK&F 107647-treated mice were adjusted to 4 × 10⁷/ml and stained with either FITC-labeled rat anti-mouse (Boehringer Mannheim, Indianapolis, IN) CD11b mAb or isotype control Ig for 30 min at 4°C. Following incubation, the cells were washed and resuspended in 2% paraformaldehyde for flow cytometry. Sample populations were analyzed for fluorescence intensity of the individual cell populations (e.g., monocytes and neutrophils) (50,000 cells) on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

**Preparation of C. albicans.** C. albicans was prepared as previously described (10). Strain B311 (serotype A; ATCC 32354) was animal passed in mice and recovered from kidney homogenates. Recovered organisms were inoculated into Sabouraud dextrose broth (SDB) and grown overnight at 37°C, and the resultant culture aliquoted and frozen at −70°C. For use in studies, organisms from thawed stock aliquots were grown overnight on Sabouraud dextrose agar (SDA) and then inoculated into SDB and incubated for 18 h at 37°C on a rotary shaker. The cells were harvested by centrifugation, washed, resuspended in normal saline, and quantified by hemocytometer. Resulting suspensions were >95% viable as determined by methylene blue dye exclusion. All inoculum sizes reported are based on viable counts as determined by CFU analysis on SDA.

**Candidacidal assay.** Two hundred C. albicans blastospores were added to quadruplicate flat-bottom microtiter wells containing 2 × 10⁷ human neutrophils (E:T = 10:1) in HBSS plus 10% autologous human sera. Various concentrations of KC proteins were added (quadruplicate wells/concentration) and following a 2-h incubation at 37°C and 5% CO₂, the plates were centrifuged, washed, resuspended in normal saline, and quantified by hemocytometer. Resulting suspensions were >95% viable as determined by methylene blue dye exclusion. All inoculum sizes reported are based on viable counts as determined by CFU analysis on SDA.

**Marine C. albicans infection model.**

**Animals.** Strictly defined flora BALB/c mice bred in an in house laminar flow barrier facility were utilized (18). The mice were housed in sterile cages and fed sterilized lab chow and water ad libitum. The Institutional Animal Care and Use Committee of SmithKline Beecham Pharmaceuticals approved procedures involving the use of laboratory animals.

**Dosing schedule.** Various concentrations of recombinant KC-T prepared in PBS were administered s.c. 2 h before infection. All mice were infected i.v. with a lethal dose of C. albicans (1.2 × 10⁶ yeasts/mouse). After infection, the animals were followed for survival. Mann-Whitney U test determined differences in survival times.

**Results.** We have previously shown that SK&F 107647 induces the production of colony stimulating activity (CSA) from stromal cell cultures in a dose-dependent fashion (19). Subsequent analysis indicated that SK&F 107647-induced CSA was not a CSF but rather a <30,000 m.w. factor that acted in synergy with suboptimal concentrations of all of the myeloid CSFs (Table I). The degree of synergy ranged from 130 to 200% of control CSF levels. The synergistic activity of SK&F 107647-induced stromal cell supernatants was best observed at suboptimal CSF concentrations, with only minor increases in CFU-GM proliferation observed in combination with optimal CSF concentrations (data not shown). Similar results were observed when supernatants were tested on human CFU-GM (data not shown).

### Table I. Stromal cell supernatant synergy with suboptimal concentrations of CSFs in murine CFU-GM assays

<table>
<thead>
<tr>
<th>CFU-GM/7.5 × 10⁵ BMC</th>
<th>CSF</th>
<th>Conc/ml</th>
<th>CSF alone</th>
<th>CSF w/supernate</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhu-M-CSF</td>
<td>20 U</td>
<td>21 ± 4</td>
<td>44 ± 2*</td>
<td></td>
</tr>
<tr>
<td>rm-GM-CSF</td>
<td>50 U</td>
<td>30 ± 1</td>
<td>62 ± 5*</td>
<td></td>
</tr>
<tr>
<td>rhu-G-CSF</td>
<td>5 ng</td>
<td>6 ± 1</td>
<td>12 ± 1*</td>
<td></td>
</tr>
<tr>
<td>rm-IL-3</td>
<td>5 ng</td>
<td>37 ± 2</td>
<td>60 ± 4*</td>
<td></td>
</tr>
</tbody>
</table>

*Marine bone marrow cells (BMC) were treated with suboptimal concentration of recombinant human (rhu) or recombinant murine (rm) CSF and 3% (v/v) SK&F 107647-stimulated C6 cell supernatants. CSF and stromal cell supernates were added to 7.5 × 10⁵ BMC in an agar CFU-GM assay. Colonies were scored after 7 days of incubation at 37°C. 7.5% CO₂ in a humidified 5% O₂ incubator. Data represent the mean CFU count of triplicate cultures ± SE.

*+, p < 0.05 vs CSF alone.

**Purification of marine HSF from the bone marrow stromal cell line, C6**

The purification of SK&F 107647-induced synergistic activity was tracked by the CFU-GM bioassay. Due to the lack of signal at 280 nm in any of the chromatography steps, no purification figures have been included.

To identify the SK&F 107647-induced synergistic factor, supernatants from peptide-treated C6 cells (40 liters) were filtered using an Omega 30-kDa m.w. cut-off cassette assembled in a Filtron Centrasette (Filtron, Northborough, MA). The filtrate was concentrated to 500 ml using a 10-kDa cassette. The retentate, which contained 100% of the synergistic biological activity, was applied to a heparin agarose column (Bio-Rad, Richmond, CA). A solution of 0.5 M NaCl was used to elute nonactive proteins from the heparin column. Bound HSF was eluted with 1 M NaCl and dialyzed in PBS before application to an Aquapore C8 RP-HPLC column (Beckman). Fractions were collected using an acetonitrile (ACN) gradient and analyzed for the presence of HSF activity on mouse CFU-GM, over an extended dilution range. The fractions containing HSF activity (41% ACN) and adjacent peaks were analyzed by amino acid sequence and mass spectrometry.

Amino acid sequence analysis of the active 41% ACN fraction identified a N-terminal sequence consistent with a four amino acid N-terminal truncated form of the murine C-X-C chemokine KC (Fig. 1). Mass spectrometry analysis of this same fraction resulted in a mass of 7459 Da, consistent with a truncated 68-residue form of KC, i.e., KC₅₋₇₂. In an adjacent peak, full-length KC₅₋₇₂ was also purified and characterized (7811 Da), but did not demonstrate any detectable hematopoietic synergistic activity on either murine or human CFU-GM in vitro.

**Purification of human HSF from the bone marrow stromal cell line, TF274**

Human SK&F 107647-induced synergistic activity was purified in a similar manner to murine HSF. Supernatants from SK&F 107647-treated TF274 cells (185 liters) were filtered with a Centrasette tangential flow concentrator (Filtron, Northborough MA) equipped with a 100K MWCO Omega-type membrane. The filtrate, 170 liters, was concentrated to 4 liters using the same apparatus equipped with a 3K MWCO Omega-type membrane. The retentate was dialyzed with PBS and adjusted to 0.5 M NaCl in PBS and applied to heparin agarose column (Bio-Rad). Bound HSF was eluted with 1.5 M NaCl in PBS (450 ml). The heparin column 1.5 M NaCl pool was concentrated to 100 ml and applied directly to an immunoaffinity column (10 ml) prepared by immobilizing anti-GROβ mAb (Austral Biologicals, San Roman CA) to Affigel 10 (Bio-Rad) at 0.5 mg/ml of wet gel. The affinity column
was developed by washing with buffer A (50 mM NaPO₄/0.15 M NaCl, pH 7.2), buffer B (5 mM NaPO₄/5 mM NaCl, pH 7.2), and buffer C (2.5 mM H₃PO₄/5 mM NaCl, pH 2.7), then re-equilibrated in buffer A. Fractions containing HSF activity determined by bioactivity on CFU-GM were pooled, reapplied to the same column, and rechromatographed. HSF activity was eluted with buffer A (50 mM NaPO₄/0.15 mM NaCl, pH 7.2) upon column re-equilibration. Fractions containing HSF activity were pooled and analyzed by N-terminal amino acid sequencing and MALDI-MS.

The observed sequence in the eluted immunoaffinity column fraction containing the HSF bioactivity was consistent with a 4-residue N-terminal truncated form of the human C-X-C chemokine GROβ (Fig. 1). Mass spectrometry indicated that this fraction contained a unique mass species of 7550 Da compared with adjacent fractions. Estimated total protein content in this fraction was 30 pmol, therefore no further purification was attempted. No full-length GROβ (expected molecular mass of 7887 Da) was found in any adjacent fractions. GROβ-T identity was distinguished from GROα and GROγ homologues by residues 20 (L in GROβ while P in GROα), 27 (K in GROβ while N in GROα and GROγ), and 29 (K in GROβ while R in GROγ). Western blotting of the GROβ affinity column acid eluate revealed a protein band migrating with recombinant GROβ (data not shown).

Hematopoietic synergistic activity of recombinant KC and GROβ proteins

KC-T and GROβ-T plasmids were constructed and proteins were expressed in E. coli and purified to confirm biological activity. Recombinant KC-T (data not shown) and GROβ-T enhanced the proliferation of murine CFU-GM stimulated by a suboptimal concentration of M-CSF (Fig. 2). Addition of recombinant GROβ-T resulted in a bell-shaped response curve similar to that observed with unpurified SK&F 107647-induced stromal cell supernatants. Significant enhancement of CFU-GM by GROβ-T was observed at concentrations between 5 pg/ml and 500 attograms/ml (p < 0.01), with 50 fg and 5 fg/ml concentrations of GROβ-T consistently resulting in a statistically significant enhancement of CFU-GM proliferation (p < 0.01; n = 6 experiments). Similar results were observed for recombinant KC-T tested on either murine or human CFU-GM (data not shown).

The comparative specific activities of full-length and truncated KC and GROβ for enhancement of CFU-GM proliferation were evaluated. A concentration range of 500 ng to 5 ag/ml of recombinant chemokines was studied with murine bone marrow cells stimulated with 20 U of M-CSF. The absolute numbers of CFU-GM that were statistically (p < 0.01) greater than the number of CFU-GM stimulated by 20 U M-CSF alone were utilized to determine the hematopoietic synergistic specific activity of each chemokine according to the formula [(CFUtest – CFU(M-CSF) + mg protein tested] / CFU(M-CSF) – CFUtest]. Full-length KC was without significant CFU-GM synergistic activity at any concentration tested up to 100 ng/ml, whereas GROβ demonstrated modest CFU-GM-enhancing capacity of ~2 × 10⁴ U/mg protein. In contrast, both KC-T and GROβ-T chemokines demonstrated dramatic increases in specific activity. In six experiments, the specific activity of GROβ-T in the CFU-GM synergy assay was ~10¹²–10¹⁵ U/mg protein, which was >10 million times more potent than full-length GROβ. A similar specific activity of 1 × 10¹³ U/mg protein was observed for recombinant KC-T.

Effect of anti-KC Abs on SK&F 107647 induced serum HSF activity in the mouse

C57BL mice were injected i.p. with 100 ng/kg SK&F 107647. A group of mice were pretreated 24 h previously with 10 ng of affinity-purified anti-KC antiserum or rabbit IgG control followed by administration of SK&F 107647. Six hours after injection, animals were sacrificed and serum prepared. Serum samples from three mice per group were fractionated on 30K MW membranes to remove serum CSF and diluted in the HSF bioassay with suboptimal

FIGURE 1. Amino acid sequence alignment of murine and human SK&F 107646-induced stromal cell-derived synergistic activity. Shown are the full-length murine KC1–72 protein (7811 Da) and the N-terminal sequence of the HSF active form (7459 Da), KC-T. The m.w. was determined by MALDI-MS and N-terminal sequence analyses are consistent with an intact C terminus of GROβ.

FIGURE 2. GROβ-T augmentation of recombinant human M-CSF (20 U/ml) induced murine CFU-GM. Various concentrations of GROβ-T were added to murine 7.5 × 10⁵ bone marrow cells in an agar CFU-GM assay. Data represent the mean increase in CFU-GM ± SE from six independent experiments.
concentrations of M-CSF. The serum from mice treated with anti-KC or the IgG control Ab did not contain any synergistic activity detectable in these assays (Fig. 3). Serum collected from IgG pretreated mice 6 h after a single injection of SK&F 107647 (100 ng/kg) serum was collected and analyzed in a CFU-GM assay in combination with suboptimal M-CSF as described in Materials and Methods. Data shown represent the 20 μl/well dilution of fractionated serum. Baseline represents with number of CFU-GM stimulated by suboptimal M-CSF alone. HSF units of activity were calculated based on the formula [(CFU-test − CFU-control)/ml of serum] (*, p < 0.01). Data are representative from several experiments with identical results.

**Effect of KC and GROβ forms on effector cell activation**

**Activity of GROβ forms on human PMN function.** To assess the activity of both truncated and full-length forms of GROβ, we tested the response of these two isoforms on calcium mobilization and chemotaxis using freshly isolated human neutrophils. Both GROβ and GROβ-T both promoted substantial calcium mobilization in PMN over the dose-response range 0.03–100 nM with EC_{50} = 0.2 and 2.2 nM for GROβ-T and GROβ, respectively (Fig. 4A). The same difference in potency was also noted for GROβ-T and GROβ in the human neutrophil chemotaxis assay, which like the calcium assay identified both ligands as full agonists. In the chemotaxis assay GROβ and GROβ-T both promoted chemotaxis with EC_{50} values of 2 and 0.3 nM, respectively (Fig. 4B). IL-8 was used for comparison and had an EC_{50} similar to GROβ-T. Thus GROβ, GROβ-T, and IL-8 were found to be chemotactic rather than chemokinetic by checkerboard analysis (data not shown).

**KC on mouse cells.** Superoxide anion release from murine peritoneal macrophages in response to recombinant KC and KC-T proteins was determined at 10-min intervals. Optimum activity was observed following 10–20 min, and the 20-min data are represented in Fig. 5. Maximal responses occurred at 0.1–1 and 100 nM with truncated and full-length KC proteins, respectively (p < 0.01). KC-T was 1,000- to 10,000-fold more potent than KC in stimulating superoxide anion release. Of note is the bell-shaped dose-response curve to KC-T, similar to the dose-response curve for CFU-GM synergistic activity.

**KC on human blood cells.** The EC_{50} for full-length KC could not be accurately determined because a maximal response was not observed at the highest concentration tested. Therefore, we compared the potency of full-length and truncated chemokine forms based on the concentrations that resulted in 150% of the control response. When human neutrophils were treated with both forms of KC, the effective concentrations for eliciting a respiratory burst were 10 nM and 0.2 pM for KC and KC-T, respectively (Fig. 6A).

**FIGURE 3.** Effect of anti-KC on SK&F 107647-induced serum synergistic activity. C57BL mice were pretreated with 10 ng of control rabbit IgG or anti-KC Ab. Six hours after a single injection of SK&F 107647 (100 ng/kg) serum was collected and analyzed in a CFU-GM assay in combination with suboptimal M-CSF as described in Materials and Methods. Data shown represent the 20 μl/well dilution of fractionated serum. Baseline represents with number of CFU-GM stimulated by suboptimal M-CSF alone. HSF units of activity were calculated based on the formula [(CFU-test − CFU-control)/ml of serum] (*, p < 0.01). Data are representative from several experiments with identical results.

**FIGURE 4.** A. Effect of GROβ-T (●) and GROβ (■) on calcium mobilization in human neutrophils. Freshly isolated neutrophils where loaded with fura-2 and stimulated with varying concentrations of either GROβ-T or GROβ. Maximal calcium mobilization was monitored and noted. The figure depicts that amount of calcium mobilized in nM at each concentration of chemokine. Each point is the average of four separate determinations ± SEM. B. Dose-dependent chemotaxis of human neutrophils to GROβ-T (●), GROβ (■) or IL-8 (○). Each point represent the average of three determinations counted in triplicate. The chemotactic index is derived from the number of cells that migrated to the chemokine divided by the number of cells which migrated in the absence of added chemokine.

**FIGURE 5.** Increased superoxide activity of murine peritoneal macrophages in response to recombinant KC and KC-T proteins. Resident murine peritoneal macrophages were stimulated with each protein. Data are presented as the mean % of control response superoxide anion production. The OD_{550} of each well was determined at 10-min intervals for up to 1 h, and the amount of cytochrome c reduced was calculated. Control superoxide activity was determined in the presence of cells and PBS. Representative data from two experiments are expressed as a mean percentage of the control (PBS) response at peak response times from triplicate wells.
Enhancement of human monocyte superoxide response has generally not been observed as a major function of KC and GRO protein (1). Consistent with this observation, human blood monocytes responded weakly to KC at high concentrations (150% of control at 100 nM). However, KC-T activated monocytes at 0.2 nM, representing a 500-fold increase in potency.

**GROβ on human cells.** The results of GROβ and GROβ-T on superoxide anion release from human blood cells were equivalent to results with KC proteins. The effective concentrations for eliciting a respiratory burst from human neutrophils were 10 nM and 0.1 pM for GROβ and GROβ-T, respectively (Fig. 6B). GROβ-T was ≥10,000-fold more potent than GROβ. Human blood monocytes responded weakly to GROβ at high concentrations (150% of control response at 30 nM). However, GROβ-T activated monocytes at 0.1 nM, representing a 300-fold increase in potency.

**Candidacidal activity.** The increased production of antimicrobial superoxide anion is a good indicator for enhanced phagocytic cell killing of organisms such as fungi. We determined the effects of recombinant KC proteins on the capacity of human neutrophils to kill *C. albicans* in vitro (Fig. 7). Candidacidal activity was measured following a 2-h coincubation of neutrophils and fungi (E:T ratio = 10:1). Treatment of neutrophils with 0.001–100 nM KC-T resulted in significantly increased levels of candidacidal activity (~1.5- to 3-fold increase, *p* < 0.01) with optimum enhancement occurring at a dose of 1 nM KC-T (225% of control response). There was a minimal but significant increase in candidacidal activity as a result of treatment with KC at doses of 10 and 100 nM (*p* < 0.05).

**Neutralization of SK&F 107647-induced activation of effector cells with anti-KC antiserum.** We have previously shown that administration of SK&F 107647 in mice increased the activity of effector cells (8, 9). Administration of 10 ng/kg for 5 days results in an increase in both the percentage of neutrophils and macrophages expressing CD11b and the intensity of CD11b expression as measured by mean channel fluorescence (MCF). In these experiments, mice were pretreated with 10 ng of either control rabbit IgG or anti-KC Ab 1 day before initiation of SK&F 107647 treatment. After 5 days of SK&F 107647 treatment, blood was collected from three mice per group and analyzed for cell surface expression of CD11b by flow cytometry. As shown in Fig. 8, administration of SK&F 107647 increased both the percentage of

**FIGURE 6.** Comparative effects of recombinant KC (A) and GROβ (B) proteins on superoxide response of human blood neutrophils and monocytes in vitro. Various concentrations of proteins were added to enriched human peripheral blood neutrophils (PMN) or monocytes (MN). The OD550 of each well was determined at 10-min intervals for up to 1-h incubation at 37°C and 5% CO2, and the amount of cytochrome c reduced/quadruplicate wells was calculated. Control superoxide activity was determined in the presence of PBS. Data are expressed as a mean percentage of the control (PBS) response at peak response times from triplicate wells.

**FIGURE 7.** The effect of recombinant KC proteins on the capacity of human neutrophils to kill *C. albicans*. Enriched human neutrophils were cultured with recombinant KC/KC-T and 200 *C. albicans* blastospores for 2 h. *Candida* CFU were visualized as described in Materials and Methods. Data represent the mean % control killing response of triplicate wells/concentration.

**FIGURE 8.** The effect of affinity purified anti-KC on SK&F 107647 induced expression of CD11b on effector cell population in vivo. C57BL mice were pretreated with control rabbit IgG or anti-KC Ab 1 day before initiation of SK&F 107647 treatment. After five daily injections of SK&F 107647, mice were bled and cells were stained for expression of CD11b. Analysis of CD11b expression on neutrophils and monocytes was performed on gated cell populations with a Becton Dickinson FACScan. Data are presented as the percentage CD11b expressing neutrophils and monocytes (top) and the mean channel fluorescence (bottom).
neutrophils and monocytes expressing CD11b (top) and also the MCF of CD11b expressing cells (bottom). Pretreatment with control IgG was without effect on SK&F 107647-induced CD11b up-regulation; however, pretreatment with anti-KC Ab completely blocked the CD11b effects of SK&F 107647 in vivo.

Analysis of KC-T in a murine C. albicans infection model

The effect of KC-T in enhancing superoxide production and candidacidal activity of neutrophils and monocytes in vitro suggested that this chemokine may have biological activity in an animal fungal infection model in which neutrophils and monocytes have been shown to be the primary host cells responsible for anti-fungal activity. We therefore investigated the effect of administration of KC-T on survival in a lethal murine C. albicans infection model. Administration of SK&F 107647 in this model effectively increases the survival of treated mice (8). Mice were dosed s.c. with various concentrations of KC-T or PBS 2 h before i.v. infection with a lethal dose of C. albicans. At 15 days postinfection, no survivors were present in the control group. In contrast, significantly enhanced survival was observed in mice treated with a single injection of 63 fg or 125 fg/kg KC-T. The response to KC-T showed a bell-shaped curve with higher (250 fg/kg) and lower (32 fg/kg) doses failing to significantly protect mice from lethal infection.

Discussion

Chemokines are proteins with numerous biological functions related to the regulation of hematopoiesis and the function of host defense effector cells, i.e., granulocytes and monocytes. The production of chemokines is elicited by a variety of stimuli including those found at sites of inflammation and infection (1, 2). Administration of the hematoregulatory peptide, SK&F 107647, exhibits anti-infective activity in animal models of candida and bacterial sepsis (9, 10). Here we demonstrate that the peptide SK&F 107647 exerts its hematoregulatory and anti-infective activities through the production of specifically truncated C-X-C chemokines: KC-T in the mouse and GROβ-T in human. More importantly, these results demonstrate the formation of specific N-terminal processed forms of KC and GROβ possessing previously unknown and extremely potent hematoregulatory activities.

Chemokines have been reported to have both enhancing and suppressive effects on hematopoietic progenitor cell proliferation (5, 6, 20). In these studies, GROβ-T and KC-T are potent synergistic factors for CFU-GM proliferation in vitro. In addition, administration of SK&F 107647 to mice results in the production of HSF activity in the serum that is neutralized by anti-KC Ab in vivo and in vitro, further supporting the physiological relevance of these chemokine forms. The molecular signaling mechanisms for this effect is currently unknown. Chemokines in the GRO family are known to bind the CXCR2 receptor (1). It has yet to be determined whether CXCR2 receptors are present on marrow hematopoietic progenitors (3, 21, 22); however, the growth of single cell CD34+ progenitors is clearly affected by chemokines (23). In the in vitro effect of C-X-C chemokines such as human IL-8 and GROβ (macrophage-inflammatory protein-2α) on progenitor cells is growth suppression (4, 24) rather than the enhanced growth observed with GROβ-T and KC-T. Full-length GROβ has CFU-GM enhancing activity, whereas full-length KC had no enhancing effect at the concentrations tested. It has been reported that the suppressive effect of IL-8 and melanoma growth stimulatory activity (GROα) on the growth of the IL-3-dependent murine myeloid progenitor cell line 32D was via the murine CXCR2 (25). In addition, GROβ-T has ~10 times the affinity for the CXC receptor than full-length GROβ and the CXCR2 receptor as defined by human neutrophil chemotaxis and Ca2+ mobilization assays. In part, the apparent increases in receptor affinity for GROβ-T may account for some of the differences in potency seen in the hematopoietic synergistic assay. The hematopoietic synergistic activity of GROβ-T is antagonized by the CXCR2 specific antagonist SB-220052 with an IC50 of 500 nM providing further evidence that the CXCR2 receptor is involved GROβ-T-mediated hematopoietic activities (data not shown and Ref. 26). Alternatively, the increased potency of GROβ-T and KC-T may indicate an indirect mechanism of action involving multiple narrow cell types whose amplified signals result in enhanced CFU-GM proliferation. The cellular mechanisms involved in the enhanced potency of truncated GROβ and KC is under investigation.

Identification of truncated chemokines has been reported; however, the magnitude of enhanced response observed with GROβ-T and KC-T on proliferation of CFU-GM and effector cell function are more dramatic than reported for truncations of any other chemokines. An N-terminal 5-aa truncated form of GROβ has been reported to be ~1 log more potent than full-length GROβ in activating neutrophil elastase release (27). A mixture of N-terminal short forms of GCP-2 has higher specific activity than longer forms as measured by neutrophil activation in vitro (28, 29). The 77-aa form of IL-8 is less potent than the shorter 72-aa form in effector cell activation and receptor binding (30). In contrast, IL-8 truncated by 4 N-terminal residues is less potent in binding to the IL-8 receptor and stimulating chemotaxis and effector cell activation (31). Recently, N-terminal truncated forms of ENA-78, GROα, and GROγ produced by tumor cells and leukocytes have demonstrated increased potency in chemotactic assays and Ca2+ signaling relative to full-length forms (32). N-terminal truncations of RANTES (33) and MCP-1 (34, 35) have also been described having equivalent or less activity compared with their respective full-length chemokines. Antagonistic N-terminally truncated forms of RANTES with no chemotactic activity, but retaining anti-HIV activity have also been described (36, 37). In many of these studies multiple N-terminal truncations of various lengths were observed during purification indicating possible nonspecific aminopeptidase cleavage.

Specific N-terminal cleavage of chemokines such as RANTES and stromal-derived-factor-1α (SDF-1α) and SDF-1β can be mediated by dipeptidyl peptidase IV (CD26). CD26-mediated cleavage of RANTES reduces activity on human monocytes (38), whereas cleavage of SDF-1α and SDF-1β abolishes both chemotactic and anti-viral activity (39, 40). Many chemokines including KC and GROβ have a proline in the second amino acid position from the N terminus. CD26 cleaves 2 aa at a time with preference...
for post-proline and post-alanine cleavage (41). Full-length KC and GROβ have proline at position 2 and alanine at position 4. During purification of SK&F 107647-stimulated stromal cell supernatants, an N-terminal 2-residue truncated form of KC2-72 was isolated; however, it lacked CFU-GM synergistic activity (data not shown). It is unclear whether the production of specific 4-aa truncated chemokines in SK&F 107647-stimulated stromal cells is mediated by CD26. It is tempting to speculate that the specific enzymatic truncation of GROβ and KC at the same amino acid position (4 N-terminal amino acids) represents an added level of specificity related to the mechanism of action of SK&F 107647.

Chemokines in the CC and CXC classes have been shown to enhance effector cell function (1). The CC chemokines, in general, have more effects on monocytes and T cells, while CXC chemokines enhance effector cell function (1). The CC chemokines, in general, (4 N-terminal amino acids) represents an added level of specificity of these activities of KC-T and GROβ-T. The concentrations of KC-T and GROβ-T that activated effector cell populations and those that enhanced CFU-GM synergistic activities were very different. Enhanced CFU-GM activity of KC-T or GROβ-T occurred at concentrations of 0.01–0.1 pM (50–500 fg/ml). Full-length KC was inactive in the CFU-GM assay, whereas GROβ was 10 million-fold less active. In human PMN studies, KC-T and GROβ-T enhanced superoxide activity at 0.1–0.2 pM compared with 10 nM for the full-length forms (10,000–50,000-fold increase in potency). In human monocytes, KC-T and GROβ-T enhanced superoxide activity at 0.1–0.2 nM, whereas the full-length forms were active at 30–100 nM (300–500-fold increase in potency). The complex mechanisms involved in differential enhanced potency with the truncated chemokines are unknown. All of the assay systems used contain different functional cell types, which may have differential sensitivities to the truncated chemokines. In addition, the response of the same cell type was differentially enhanced potency with the truncated chemokines are unknown. Differential enhanced potency with the truncated chemokines are unknown. All of the assay systems used contain different functional cell types, which may have differential sensitivities to the truncated chemokines. Agents that can increase host defense effector cell activity, such as bacillus Calmette-Guerin (42, 43) M-CSF (44), GM-CSF (45), and SK&F 107647 (9) have all demonstrated protection in experimental models of C. albicans fungal infection. Mechanistically, we have demonstrated that the in vitro and in vivo effects of SK&F 107647 are mediated by KC-T production in the mouse. Studies were performed to determine whether enhanced effector cell function (enhanced superoxide and candidacial activity) elicited by KC-T in vitro could result in protection of mice challenged with lethal doses of C. albicans. KC-T demonstrated potent anti-infective properties in this model in the 60- to 120-fg/kg dose range. KC-T mimicked the effectiveness of SK&F 107647 in vivo and also demonstrated a bell-shaped dose response that is characteristic of hematoregulatory compounds (9, 10, 44). The mechanism for this bell-shaped dose response is presently unknown. This study is the first demonstration of in vivo efficacy of a CXC chemokine in an animal infectious disease model.

To our knowledge, this report represents the first example where any form of KC or GROβ were purified from marrow stromal cells. The discovery that truncated forms of chemokines can demonstrate dramatically enhanced potency and activity has significant implications in defining the role of chemokines in physiological and pathological conditions.

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References


