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Rabbit neutrophil chemotactic protein (NCP) activates both CXCR1 and CXCR2 and is the functional homologue for human CXCL6

Julie Catusse, Sofie Struyf, Anja Wuyts, Myke Weyler, Tamara Loos, Klara Gijsbers, Mieke Gouwy, Paul Proost, Jo Van Damme*

Laboratory of Molecular Immunology, Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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Abstract

Neutrophil chemotactic protein (NCP) is a rabbit CXC chemokine with activating and chemotactic properties on neutrophilic granulocytes. Although its selective activity on neutrophils is demonstrated, its interactions with specific chemokine receptors are not defined. For further functional characterization, NCP was chemically synthesized and was found to be equipotent as natural NCP in neutrophil chemotaxis. To identify its human homologue, we separately expressed two potential rabbit NCP receptors (CXCR1 and CXCR2) in Jurkat cells. Pure synthetic NCP was equally efficient to promote chemotaxis through either rabbit CXCR1 or CXCR2. Moreover, chemotaxis assays on rabbit CXCR1 and CXCR2 transfectants showed that NCP uses the same receptors as interleukin-8 (IL-8), a major rabbit CXC chemokine, but not rabbit GROα, which only recognized CXCR2. In addition, specific inhibitors for CXCR1 or CXCR2 reduced rabbit neutrophil chemotaxis induced by NCP and rabbit IL-8. Furthermore, NCP and the structurally related human CXCR1/CXCR2 agonist CXCL6/GCP-2 (granulocyte chemotactic protein-2) cross-desensitized each other in intracellular calcium release assays on human neutrophils, further indicating that both chemokines share the same receptors. The inflammatory role of NCP was also evidenced by its potent granulocytosis inducing capacity in rabbits upon systemic administration. This study provides in vitro and in vivo evidences that NCP is the functional rabbit homologue for human CXCL6/GCP-2 rather than the most related CXCR2 agonist CXCL5/ENA-78 (epithelial cell-derived neutrophil activating peptide-78). It is concluded that the rabbit is a better model to study human neutrophil activation compared to mice, which lack CXCL8/IL-8.

Keywords: Rabbit; Chemokine; Neutrophils; Receptor; Chemotaxis; Granulocytosis

1. Introduction

Chemokines are a family of small polypeptides implicated in the localization and activation of leukocytes. These chemotactic cytokines were originally identified for their ability to recruit leukocytes during inflammation and immune responses. Their central position in innate and adaptive immunity, as well as their ability to attract non-immune cells, implicates chemokines in several pathophysiological conditions (organogenesis, tumorigenesis, acute and chronic inflammation, etc.)[1–3]. Therefore, chemokines and their G-protein-coupled receptors represent a target of interest in pharmaceutical research, and animal models for the various chemokine-related diseases are extensively investigated [4–6].

Chemokines have been classified in four different families, depending on the position of their first two cysteines. Chemokines bearing the ELR (for Glutamate-Leucine-Arginine) and CXC (for Cysteine-X-Cysteine) sequences are specialized in the chemoattraction and activation of neutrophils. Seven ELR$^+$-CXC chemokines, which all selectively bind to receptor CXCR2 are found in human: interleukin-8 (IL-8 or CXCL8 in the systematic nomenclature), granulocyte chemotactic protein-2 (GCP-2/CXCL6), growth-related oncogen-α, β, γ (GROα, β, γ/CXCL1, 2, 3), neutrophil-activating peptide-2 (NAP-

Abbreviations: ENA-78, epithelial cell-derived neutrophil activating peptide-78; Fmoc, fluorenylmethyloxycarbonyl; GCP-2, granulocyte chemotactic protein-2; GRO, growth related oncogen; IL-8, interleukin-8; NCP, neutrophil chemotactic protein; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoro acetic acid; SEM, standard error of the mean

* Corresponding author. Tel.: +32 16 33 7348; fax: +32 16 33 7340.
E-mail address: jozef.vandamme@rega.kuleuven.ac.be (J.V. Damme).

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2/CXCL7) and epithelial cell-derived neutrophil-activating peptide-78 (ENA-78/CXCL5). However, IL-8 and GCP-2 also activate CXCR1. Human CXCR1 and CXCR2 share 73% identity at the amino acid level and both are strongly expressed at the surface of neutrophils [7]. Nevertheless, cells activated via chemokine binding to one or the other receptor show different responses, even after activation by the same chemokine [8]. Several CXCR2 antagonists have been characterized so far and chemokine and receptor systems have been investigated in different species [9–11]. Most species (monkey, rabbit, rat, etc.) possess homologous ELR$^+$-CXC chemokines (e.g., IL-8, GROα, etc.) and receptors (e.g., CXCR1 and CXCR2) [12–14]. But, some clear differences exist in the number of chemokines encoded by the genome of different species. For example, mice lack a direct equivalent for IL-8, the key human ELR$^+$-CXC chemokine. Moreover, mouse, guinea pig, dog and cow do possess CXCR2, but the equivalent receptor for CXCR1 still needs to be identified [15–17].

NCP is a recently discovered rabbit chemokine that presents high sequence homology with both human ENA-78 and GCP-2 (82 and 72% amino acids sequence identity, respectively) [18–20]. These two closely related human ELR$^+$-CXC chemokines activate neutrophils, but ENA-78 is specific for CXCR2, whereas GCP-2 binds both CXCR1 and CXCR2. The present study was designed to further characterize NCP and to find out whether it is the functional homologue of GCP-2 or ENA-78. We have therefore proceeded to its chemical synthesis and have compared its in vivo and in vitro potencies with the natural form. Synthetic NCP was then used to investigate its activity on rabbit CXCR1- or CXCR2-transfected cell lines and to compare NCP with the rabbit homologues for human IL-8 and GROα. It was demonstrated that, like rabbit IL-8, NCP activates both CXCR1 and CXCR2; which allows to conclude that NCP is the functional and structural homologue of human GCP-2.

2. Materials and methods

2.1. Synthesis and purification of NCP

NCP was prepared by solid-phase peptide synthesis. Amino acids with fluorenylmethyloxycarbonyl (Fmoc) protected α-amino groups were used to proceed to standard FastMoc programs on a Model 433 A peptide synthesizer (Applied Biosystems) as described previously [21]. Final deprotection and cleavage of the peptide from the resin was performed with trifluoro acetic acid (TFA), and the synthetic chemokine was separated from the resin by filtration through a medium porosity glass filter. Crude synthetic NCP was separated from incompletely synthesized peptide by reversed-phase high performance liquid chromatography (RP-HPLC) on a Resource RPC column (Amersham Pharmacia Biotech). After purification, disulfide bridges were formed by incubation (90 min, 20 °C) of unfolded peptide in 150 mM Tris, pH 8.7, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.3 mM oxidized glutathione, 3 mM reduced glutathione and 1 M guanidinium chloride. The folded peptide was purified by RP-HPLC (220 × 2.1 mm C8 Aquapore RP-300 column, Applied Biosystems) and eluted in acetonitrile (0–80% (v/v) acetonitrile gradient in 0.1% (v/v) TFA; 0.4 ml/min, 0.4 ml/fractions). The molecular mass of the folded peptide was confirmed by mass spectrometry on an Esquire ion trap mass spectrometer (Esquire-LC, Bruker Daltonics).

2.2. Reagents

Natural rabbit IL-8 was produced from lipopolysaccharide-stimulated rabbit alveolar macrophages and purified to homogeneity as previously described [18]. Recombinant human GCP-2 was purified from the periplasm of *Escherichia coli* [22]. Natural human IL-8 was purified to homogeneity from monocyte-derived conditioned medium [23]. Recombinant rabbit GROα (1–72) was a gift from Prof. T.R. Martin (Seattle Veterans' Affairs Medical Centre).

2.3. Granulocytosis induction

Granulocytosis was induced in New Zealand white rabbits (± 3 kg) as described previously [23,24]. Natural rabbit IL-8 (1.6 μg/kg) or synthetic NCP (8.8 μg/kg) were injected (1 ml) intravenously. Blood samples were collected (1 ml) in heparinized tubes at several time intervals after treatment by bleeding at a peripheral ear vein. The total number of cells was counted microscopically with a Bürker chamber. The percentage of granulocytes was evaluated microscopically after staining with Hemacolor solutions (Merck). Control rabbits injected with buffer (0.9% NaCl) did not show significant changes in blood cell counts. The chemokine preparations were devoid of an active endotoxin concentration (<1.5 pg endotoxin per dose injected) as determined by the *Limulus amebocyte* lysate assay.

2.4. Cells and flow cytometry

Granulocytes were isolated from fresh rabbit or human blood by Lymphoprep density gradient centrifugation (Invitrogen) for 20 min at 400 × *g*. Erythrocytes from the granulocyte pellet were removed by sedimentation for 25 min at 37 °C in hydroxyethyl-starch solution (Plasmasteril; Fresenius AG). Remaining erythrocytes were lysed by a hypotonic shock (30 s) in bi-distilled water.

Rabbit CXCR1 and CXCR2 cDNA were cloned from rabbit neutrophils. Cloning was realized with primer couples designed on published rabbit CXCR1 and CXCR2

sequences [13,25]. DNA sequences were inserted into the pcDNA-3.1 plasmid (Invitrogen). After sequencing, these were used to transfect Jurkat T cells (ATCC: TIB-152) by DMRIE-C Reagent (Invitrogen) according to the manufacturer's protocol. Cells were grown in RPMI 1640 supplemented with non-essential amino acids and glutamine solutions and 10% (v/v) foetal bovine serum (Invitrogen). G418 was added to the medium (500 μg/ml) as a selection agent (Invitrogen).

The receptor expression levels were checked by flow cytometry analysis. Briefly, receptor transfected cells (10^6) were stained (30 min, 4 °C) with fluorescein isothiocyanate conjugated anti-human CXCR1 or R-phycoerythrine conjugated anti-human CXCR2 antibodies or relevant labelled isotype control antibody (PharMingen). Samples were analyzed on FACScalibur (Becton Dickinson) using CellQuest software (Becton Dickinson).

2.5. Chemotaxis assay

The chemotactic activity of different chemokines was determined by a 48-well Boyden chamber assay as described previously [24]. Briefly, the lower compartments of the chamber were filled with chemokine appropriately diluted in Hanks' balanced salt solution (Invitrogen) completed with 1 mg/ml human serum albumin (Belgian Red Cross), or with buffer alone as negative control. Each chemokine dilution was tested in triplicate. The upper compartment of the chamber was filled with receptor-transfected Jurkat T cells or freshly isolated rabbit neutrophils, previously washed in dilution buffer, and suspended at a final concentration of 2×10^6 cells/ml. Cell migration between the two compartments, through a 5 μm pore-size polycarbonate membrane (Nuclepore, Corning Costar), was allowed for 45 min or 4 h for neutrophils or Jurkat cells, respectively. After incubation, the cells were fixed and stained using Hemacolor solutions (Merck). Migrated cells were counted microscopically in 10 oil immersion fields at a 500× magnification. The chemotactic index was defined as the ratio of cells migrated toward the chemokine versus the cell numbers obtained with the buffer. Inhibition experiments were carried out as previously described [26]. Briefly, cells were incubated for 30 min at 4 °C with 10 μg/ml of the anti-human CXCR1 antibody (R&D Systems) or with 100 nM of the CXCR2 antagonist SB 225002 (Calbiochem–Novabiochem). The chemotaxis indexes were calculated by dividing the number of migrated cells in response to chemokine in the presence of antagonist by the number of migrated cells in the presence of the antagonist alone.

2.6. Calcium assay

Intracellular calcium release was measured by monitoring intracellular calcium concentration ($[Ca^{2+}]_i$) by fluorescence spectrometry as previously described [27]. Briefly, cells were loaded with the fluorescent dye fura-2. Upon excitation at 340 and 380 nm, fura-2 fluorescence was measured at 510 nm in an LS50B luminescence spectrophotometer (PerkinElmer). The $[Ca^{2+}]_i$ was calculated from the Grynkiewicz equation with a K_d of 224 nmol/l [28].

3. Results

3.1. Chemical synthesis of rabbit NCP (8–76) and testing of biological activity

Natural NCP from alveolar macrophages could be generated only in limited amounts and the purified preparations showed heterogeneity due to post-translational processing. Furthermore, NH_2-terminal truncation of NCP resulted in enhanced neutrophil chemotactic activity [18]. For these reasons, the most predominant and active naturally processed form of NCP, i.e. NCP (8–76), missing the first seven NH_2-terminal amino acids, was chemically synthesized by Fmoc chemistry using the 0.1 mmol scale. After synthesis, NCP (8–76) was deprotected, folded and purified to homogeneity following a procedure described for other chemokines synthesized in our laboratory [21,29]. Fig. 1 illustrates the RP-HPLC profile of folded NCP (8–76), which peaked around 25% acetonitrile in the elution gradient. Both NH_2-terminal sequencing and mass spectrometry analysis confirmed the authenticity of the synthetic protein. In particular, the folded synthetic NCP (8–76) eluting in fractions 49–55 upon RP-HPLC showed a molecular mass between 7492.7 and 7494.4 that corresponds well to its theoretical molecular mass of 7492.9. In analogy to natural NCP, folded synthetic NCP (8–76) occurred as a 6-kDa monomer upon SDS-PAGE under non-reducing conditions (data not shown). Most importantly, the NCP (8–76) protein peak from RP-HPLC coin-

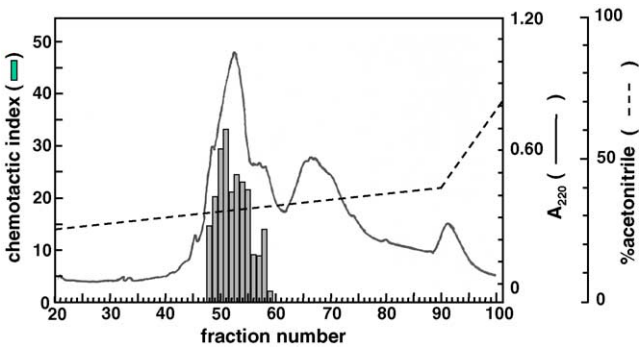

Fig. 1. Reversed-phase HPLC purification of synthetic rabbit NCP (8–76) and biological activity in the chemotaxis assay. Synthetic NCP (8–76) was injected on a 220 × 2.1 mm C8 Aquapore RP-300 column and eluted with an acetonitrile gradient (- - -). Absorbance (—) was measured at 220 nm. After incubation to allow formation of disulfide bridges, fractions (1/1000 dilution) derived from this chromatography were tested in the microchamber assay for chemotactic activity on rabbit neutrophils. Chemotactic activity is expressed as chemotactic index.

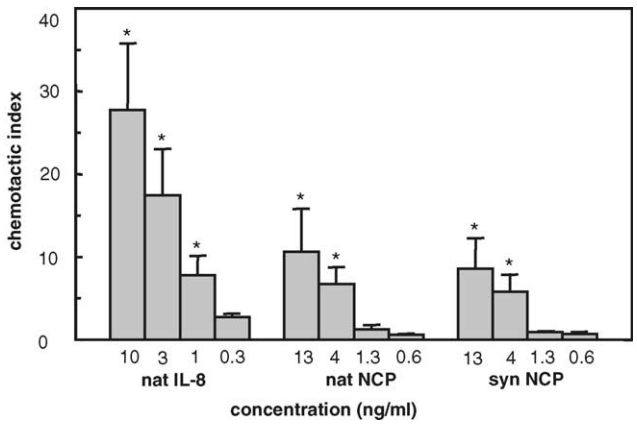

Fig. 2. Comparison of the chemotactic potency of synthetic NCP with natural forms of rabbit NCP and IL-8 on rabbit neutrophils. The dose-dependent chemotactic activity of pure natural (nat) and synthetic (syn) NCP (8–76) was compared with that of pure natural rabbit IL-8 in the Boyden microchamber assay. The activity is expressed as chemotactic indexes. Data represent the mean ± SEM of four independent experiments. Statistically significant induced chemotaxis compared to buffer as determined with the Mann–Whitney *U*-test is indicated by asterisks ($^{*}P < 0.05$). The number of randomly migrated cells in 10 microscopic fields was 4.62 ± 1.8.

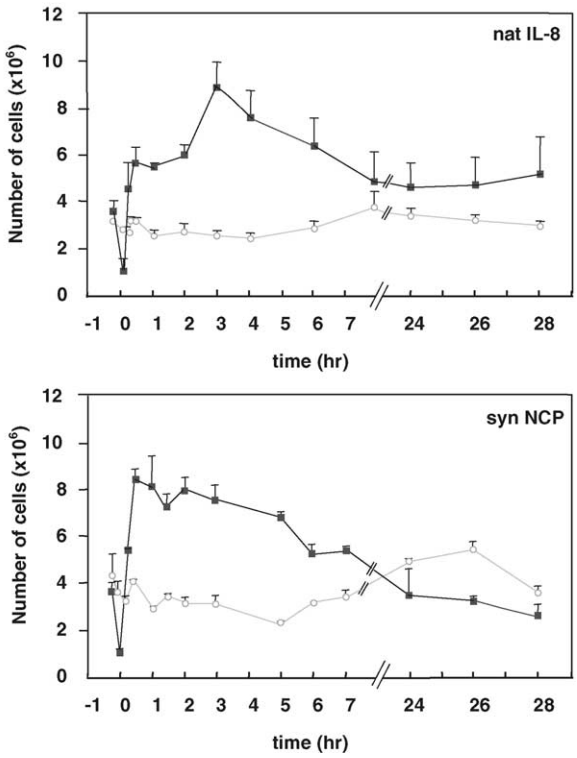

Fig. 3. Induction of granulocytosis by natural rabbit IL-8 and synthetic NCP. Rabbits were injected intravenously with 1.6 μg/kg rabbit IL-8 (upper panel) or 8.8 μg/kg NCP (8–76) (lower panel). Blood samples were taken at different time intervals. Two individuals independently evaluated the blood samples (total and differential cell counts), in triplicate. Values shown are means ± SEM of total granulocyte counts (■) and total lymphocyte counts (○). Basal blood cell levels were determined 5 min before the injection of the chemokine.

cided with potent chemotactic activity for neutrophils in the Boyden microchamber assay (Fig. 1).

3.2. Comparison of the chemotactic activity of synthetic NCP with natural forms of NCP and rabbit IL-8 on rabbit neutrophils

The chemotactic activity of the synthesized NCP (8–76) was compared to the activity mediated by natural forms of rabbit IL-8 and NCP in the Boyden microchamber assay (Fig. 2). No significant differences were seen between synthetic NCP (8–76) and a natural mixture of NCP (7–76) and NCP (8–76). Their minimal effective concentration was identical (4 ng/ml) but slightly higher than that of natural rabbit IL-8 (1 ng/ml). Synthetic NCP (8–76) was therefore used for further biological characterization of the chemokine.

3.3. Induction of granulocytosis by IL-8 and NCP in rabbits

The in vivo activity of synthetic NCP (8–76) was investigated through induction of granulocytosis upon systemic application in rabbits. As previously shown with human IL-8 [23], an immediate but transient neutropenia followed by a recruitment of neutrophils was observed after intravenous injection of natural rabbit IL-8 (1.6 μg/kg) or synthetic NCP (8.8 μg/kg). This granulocytosis peaked between 1–4 h post injection and basal blood cell counts were restored after 24 h. At the doses injected, rabbit IL-8 and NCP (8–76) induced a granulocytosis of the same magnitude, i.e. a 2-fold increase in circulating granulocyte numbers (Fig. 3). Lymphocyte (Fig. 3) and monocyte counts (data not shown) did not show any significant variation after chemokine treatment.

3.4. Expression of rabbit CXCR1 and CXCR2 in Jurkat T cells

To express rabbit CXCR1 and CXCR2 cDNA in Jurkat T cells, these cDNAs were cloned into the expression vector pcDNA-3.1. The resulting vectors or the empty pcDNA-3.1 vector were then introduced in Jurkat T cells by DMRIE-C reagent. The receptor expression levels were controlled by flow cytometry analysis by comparison with mock-transfected cells (Fig. 4). These receptor-transfected cells were subsequently used for in vitro chemotaxis experiments.

3.5. Rabbit NCP, but not rabbit GROα, uses both rabbit CXCR1 and CXCR2 for chemotaxis

In order to control whether the activity of NCP (8–76) was mediated through rabbit CXCR1 or CXCR2, it was assessed whether this ELR^{+}-CXC chemokine attracted

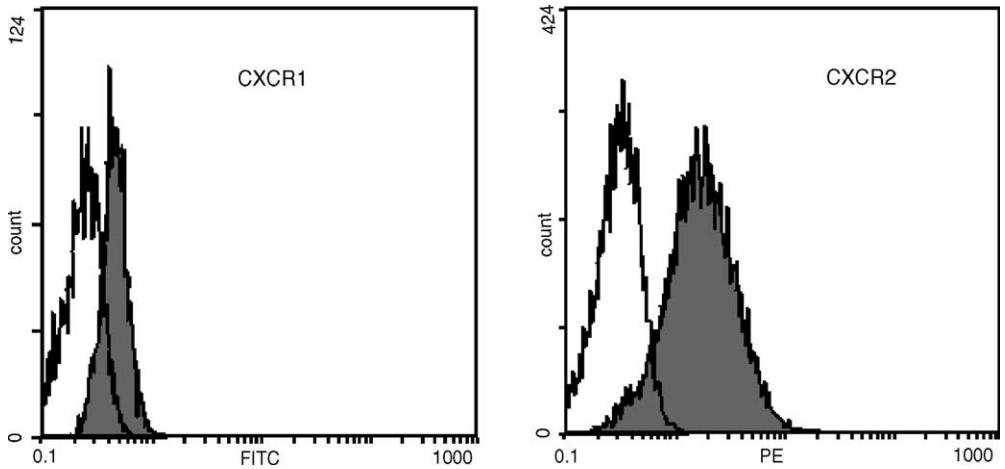

Fig. 4. Control of the expression of rabbit CXCR1 and CXCR2 expressed at the surface of Jurkat T cells by flow cytometry analysis. Cells were washed in PBS containing 1% bovine serum albumin and incubated with appropriate antibodies in the presence of non-specific IgG. The staining patterns of the CXCR1 and CXCR2 antibodies are shown in grey, the negative controls (pcDNA-3.1 mock-transfected cells) in white.

Jurkat T cells transfected with one or the other receptor. The obtained chemotaxis results are in agreement with those published for human ELR$^+$-CXC chemokines [30]. Indeed, rabbit IL-8 was able to significantly attract cells expressing either CXCR1 or CXCR2, with the highest efficiency for CXCR2 (Fig. 5). Rabbit GROα was only efficient in attracting CXCR2-expressing cells. Significantly increased chemotactic indexes (compared to mock transfected cells) were reached for NCP (8–76) (at 100 ng/ml and 10 ng/ml) with either CXCR1-or CXCR2-transfected cells. Thus, NCP (8–76) is the second rabbit CXC chemokine activating CXCR1 and CXCR2, and therefore displays a similar receptor recognition pattern as the structurally related human GCP-2 [29].

3.6. Rabbit NCP-induced chemotaxis is mediated by CXCR1 and CXCR2 on rabbit neutrophils

To confirm CXCR1 and CXCR2 usage by NCP under natural conditions, we used neutralizing antibody raised against human CXCR1, and a CXCR2 specific non-peptide antagonist, SB 225002 [10] in chemotaxis assays with rabbit neutrophils (Fig. 6). Anti-CXCR1 antibody inhibited efficiently chemotaxis mediated by both rabbit IL-8

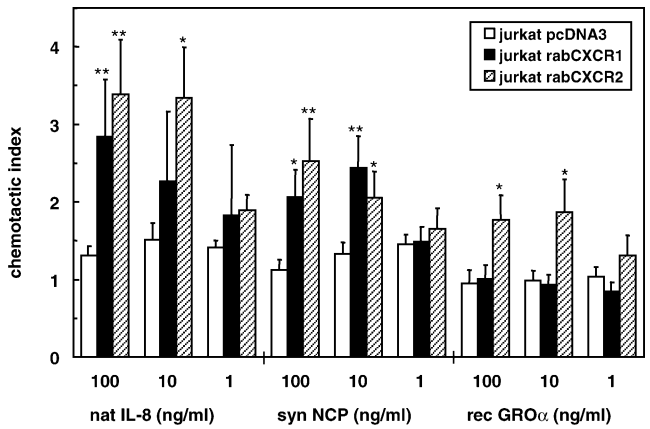

Fig. 5. Rabbit NCP (8–76), but not rabbit GROα, chemoattracts cells via both CXCR1 and CXCR2. The chemotactic potency of natural (nat) rabbit IL-8, recombinant (rec) rabbit GROα and synthetic (syn) NCP (8–76) were determined in the Boyden microchamber assay using CXCR1 or CXCR2 transfected Jurkat T cells. Results represent the mean chemotactic index ± SEM of 10 or more independent experiments (each performed in triplicate). Statistically significant differences between chemotactic indexes obtained with CXCR1 or CXCR2 transfectants compared to pcDNA-3.1 mock transfected Jurkat T cells were calculated using the Mann–Whitney *U*-test ($^*P < 0.05$, $^{**}P < 0.001$). The number of randomly migrated cells in 10 microscopic fields were for mock-, CXCR1- and CXCR2-transfected cells: 12.3 ± 2.25, 9.5 ± 1.5 and 7.06 ± 1.4, respectively.

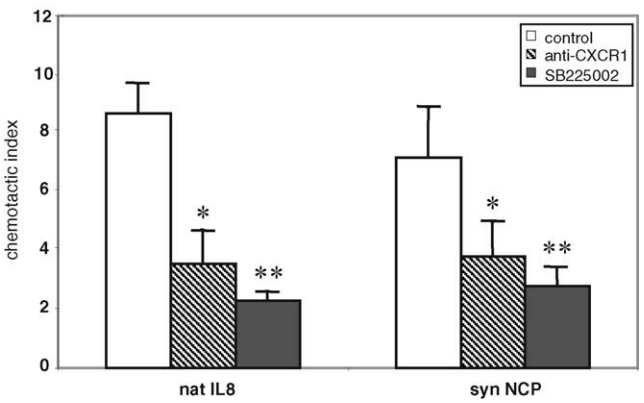

Fig. 6. Anti-CXCR1 antibody and non-peptide antagonist for CXCR2 inhibit chemotaxis induced by rabbit IL-8 and NCP. Rabbit neutrophils were incubated for 30 min at 4 °C with anti-CXCR1, antibody or a CXCR2 antagonist (SB 225002) before proceeding to the chemotaxis assay. Natural (nat) rabbit IL-8 (10 ng/ml) and synthetic (syn) NCP (20 ng/ml) induced a significant chemotactic index, which was significantly inhibited by the addition of either anti-CXCR1 antiboby (10 μg/ml) or SB 225002 (100 nM). The chemotactic response is expressed as the mean chemotactic index ± SEM, derived from five experiments. No differences were seen between the migration index of non-stimulated cells in presence or in absence of the different inhibitors. Statistically significant reduction of the chemotactic responses in presence of inhibitors are calculated by the Mann–Whitney *U*-test and are indicated by asterisks ($^*P < 0.05$, $^{**}P < 0.01$).

(10 ng/ml) and NCP (20 ng/ml). SB 225002 also reduced significantly the chemotactic activity of both the chemokines. Together these results show that CXCR1 and CXCR2 expressed at the surface of rabbit neutrophils are natural receptors for NCP and are both implicated in neutrophil migration.

3.7. Rabbit NCP desensitizes human CXCL6/GCP-2 signalling in human neutrophils

Calcium release desensitization experiments were done to further compare the NH_2-terminally processed forms of human GCP-2 (9–77) and rabbit NCP (8–76). As shown in Fig. 7 and Table 1, NCP (8–76) was able to desensitize GCP-2 (9–77)-induced intracellular Ca^{2+} releases in a concentration-dependent manner. At 25 ng/ml, GCP-2 induced a significant Ca^{2+} release in neutrophils. In two out of six independent experiments, this response was completely inhibited by a pre-treatment with 10 ng/ml of NCP (8–76) (Table 1) resulting in a mean inhibition rate of 89%. In four out of five experiments, 100% inhibition of the Ca^{2+} release induced by GCP-2 (25 ng/ml) was obtained after a pre-treatment with 40 ng/ml of NCP (8–76), with a mean inhibition of 98%. Next, inhibition of NCP (8–76) induced intracellular Ca^{2+} release was investigated. A dose–response inhibition of NCP (8–76) by GCP-2 (9–77) was also obtained. Neutrophils stimulated with 10 ng/ml of NCP (8–76) showed a Ca^{2+} release with a magnitude corresponding to that observed with 25 ng/ml GCP-2 (9–77) (data not shown). A total inhibition of this NCP (8–76) response was reached in two out of four experiments executed with a pre-treatment with 50 ng/ml of GCP-2 (mean inhibition rate of 78%). When neutrophils were pre-treated with 25 ng/ml of GCP-2, 100% inhibition was observed only in one out of four experiments, resulting in a mean inhibition rate of 70%. By lowering the GCP-2 concentration used for pre-stimulation to 1 ng/ml, the mean inhibition rates decreased to reach 4.5%. As a control, it was confirmed that human GROα and ENA-78 could not completely inhibit Ca^{2+} signalling induced by human GCP-2 (9–77) and rabbit NCP (8–76) (data not shown). In conclusion, these results indicate that most probably human GCP-2 and rabbit NCP induce Ca^{2+} signalling via the same receptors.

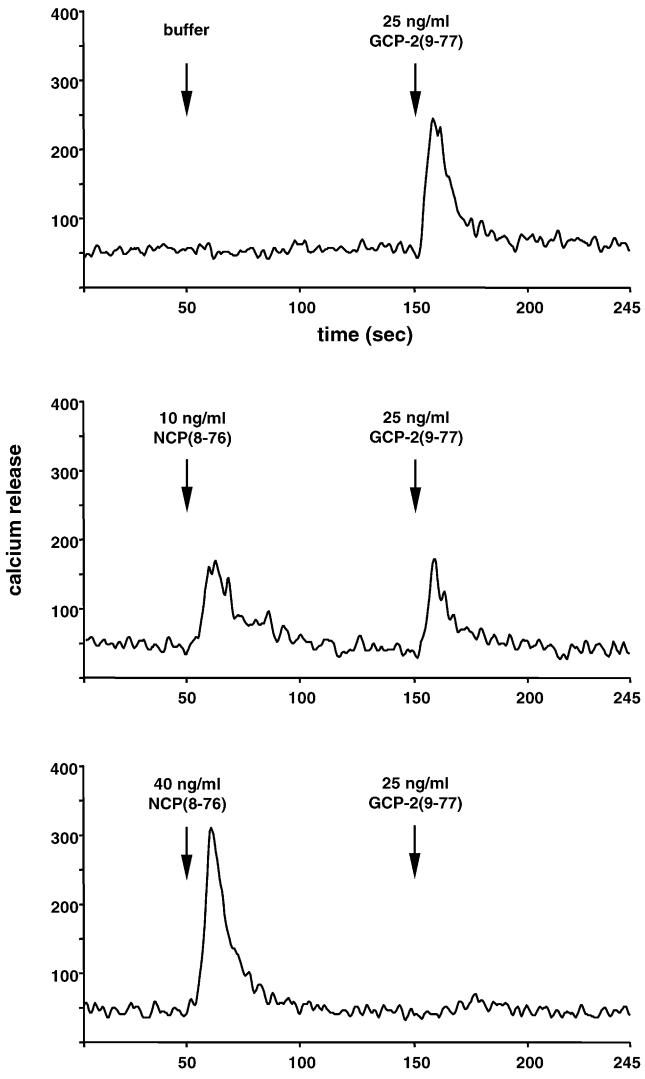

Fig. 7. Cross-desensitization of human GCP-2 (9–77) induced calcium release (nM) by rabbit NCP (8–76) on human neutrophils. Neutrophils were loaded with the calcium indicator fura-2 and exposed to a first stimulation either with buffer or increasing concentrations of rabbit NCP. Cells were then re-challenged, 100 s later, with a constant second concentration of human GCP-2 (9–77) (25 ng/ml). Results of one representative experiment are shown.

4. Discussion

Rabbit NCP has been discovered by its ability to induce chemotaxis and intracellular calcium release in neutrophils [18]. These observations together with its protein sequence allowed us to assume that rabbit NCP was a homologue for either human GCP-2 or human ENA-78. Despite their high sequence homology, these two human chemokines do not share the same receptor activation profile [30] in that ENA-78 chemoattracts cells via CXCR2, whereas GCP-2 signals through both CXCR1 and CXCR2 [29]. Although both receptors have often been associated with the progress of specific pathologies [31,32], it seems more and more evident that these two related ELR^+-CXC chemokine receptors are distinctly implicated in several diseases. For example, CXCR1, but not CXCR2, has been associated with lymphoproliferative diseases [33]. It has recently been shown that strong differences exist in CXCR1 and CXCR2 tissular brain expression [34] and ENA-78 and GCP-2 present different expression regulation pathways [33]. In this study, we have proceeded to chemical synthesis of NCP in order to identify its receptors. In agreement with several observations on chemokine processing [35–38], it has been shown that N-terminal truncation of NCP potentiates its activity [18]. Therefore, naturally occurring, trun-

Table 1
Rabbit NCP cross-desensitizes GCP-2-mediated intracellular calcium releases in human neutrophils

First stimulus	Concentration (ng/ml)	Second stimulus	Concentration (ng/ml)	% Inhibition of response to second stimulus, mean ± SEM (*n*)
NCP (8–76)	40	GCP-2 (9–77)	25	98 ± 2.2 (5)
	10		25	89 ± 4.0 (6)
	3		25	40 ± 10.7 (6)
	1		25	5.5 ± 5.5 (4)
GCP-2 (9–77)	50	NCP (8–76)	10	78 ± 16.3 (4)
	25		10	70 ± 11.3 (4)
	10		10	48 ± 15.3 (4)
	5		10	28 ± 14.5 (3)
	1		10	4.5 ± 4.5 (2)

Human neutrophils (1×10^6 cells/ml) were loaded with the calcium indicator fura-2 and exposed to a first variable concentration of human GCP-2 (9–77) or rabbit NCP (8–76). Cells were re-challenged 100 s later with a fixed concentration of the second chemokine as indicated. Values are the mean ± SEM of *n* independent experiments.

cated NCP (8–76) was chemically synthesized. The molecular mass of the obtained polypeptide was in agreement with the theoretical one. We have subsequently compared the activity of this synthetic chemokine with its natural counterpart in the in vitro Boyden chemotaxis assay on neutrophils and no significant differences were observed. Furthermore, synthetic NCP (8–76) was demonstrated to induce granulocytosis in rabbits to demonstrate its in vivo relevance and to compare its potency with that of natural rabbit IL-8. Both chemokines provoked a two-fold increase of the basal granulocyte count within 1–4 h after injection, confirming earlier observations with human IL-8 [23]. These in vitro and in vivo experiments also confirmed that NCP is a somewhat less potent neutrophil chemoattractant than rabbit IL-8 and allowed us to conclude that chemical synthesis was an efficient alternative to the time-consuming process of purifying natural protein from conditioned medium.

In addition, our study provides the first experimental proof that NCP can be considered as the rabbit equivalent of human GCP-2. Indeed, our results show that, using Jurkat cells that we have transfected with rabbit CXCR1 or CXCR2, the chemotactic activity of rabbit NCP is, like GCP-2, mediated through both CXCR1 and CXCR2 [30], and that NCP is not the functional homologue for human ENA-78. This was further confirmed by cross-desensitization experiments comparing the calcium mobilization activity of rabbit NCP with that of human GCP-2. On human neutrophils, rabbit NCP and human GCP-2 were found to have a similar efficiency in the calcium assay. Moreover, NCP was able to totally inhibit the calcium release induced by GCP-2 and vice versa. This complete inhibition of GCP-2-induced calcium release in human neutrophils by NCP pre-stimulation is an indication for the interactions between NCP and both human CXCR1 and CXCR2. Furthermore, we confirmed the implication of both receptors in rabbit neutrophil migration by inhibiting chemotaxis induced by NCP with specific inhibitors of CXCR1 or CXCR2. At 10 μg/ml, a dose that has been shown to be efficient on human cells [39], anti-human-CXCR1 neutralizing antibody inhibited efficiently chemotaxis induced by both rabbit IL-8 and NCP. Alternatively, the selective non-peptide antagonist for CXCR2, namely SB 225002, has been shown to inhibit specifically binding of IL-8 on CXCR2 and the margination on rabbit neutrophils induced by IL-8 in vivo [10]. In our hands, SB 225002 also inhibited chemotaxis of rabbit neutrophils induced by rabbit IL-8 and NCP. Our data suggest that both receptors are implicated in the migration of neutrophils induced by rabbit IL-8 and NCP.

To our knowledge, the activity of rabbit IL-8 and GROα has also never been tested on cells expressing one of these two rabbit receptors, since the activities of both chemokines were characterized using rabbit neutrophils only [40,41]. Some differences were observed in the receptor affinity of the tested rabbit chemokines. As expected from studies with human chemokines [42], we showed that rabbit IL-8 was the most potent neutrophil agonist and had stronger activity on CXCR2 than on CXCR1, whereas rabbit GROα promoted only CXCR2-mediated chemotaxis. In conclusion, we here report that NCP is the rabbit homologue for human CXCL6/GCP-2 and that rabbit IL-8 and GROα behave like their human counterparts, in that rabbit IL-8 can activate both rabbit CXCR1 and CXCR2 and rabbit GROα is specific for CXCR2. Mouse GCP-2 has proven its specific role in neutrophil recruitment by replacing the missing human IL-8 homologue. In rabbit, it seems that NCP, in addition to IL-8, also plays a role of particular importance in neutrophil activation and chemotaxis. As a consequence, with regard to neutrophil-linked pathologies, the rabbit is a more relevant model than the mouse to study inflammatory responses mediated by neutrophil-derived modulators such as proteases [35,37].

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