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Functional Distinction between CXC Chemokines, Interleukin-8 (IL-8), and Growth Related Oncogene (GRO)α in Neutrophil Infiltration

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SUMMARY: Interleukin-8 (IL-8: CXCL8) and growth related oncogene α (GROα: CXCL1) are members of the CXC chemokines. In the present study, we explored the functional distinction between these CXC chemokines in the regulation of neutrophil infiltration. Injection of either rabbit IL-8 or GROα (10 μg each) into rabbit knee joints resulted in a massive neutrophil infiltration in the joints. At their peak time point (6 hours), the number of neutrophils induced by IL-8 was more than that induced by GROα. Each chemokine induced the other chemokine in the joints. TNFα activity was induced in the joints after administration of GROα, but not IL-8. Treatment with anti-GROα mAb and/or anti-TNFα mAb failed to inhibit IL-8-induced neutrophil infiltration. In contrast, either anti-IL-8 IgG or anti-TNFα mAb decreased GROα-induced response, and the inhibition was further enhanced by coadministration of these antibodies. Thus, it appears that IL-8 acts directly, whereas GROα acts indirectly, in part, on neutrophil infiltration. The distinct difference in TNFα production between IL-8 and GROα was further investigated. In vitro, GROα induced TNFα activity in cultured synovial cells, the cells producing TNFα in the joints after GROα-injection. However, IL-8 failed to produce TNFα activity from the cells, although equivalent levels of the mRNA expression were induced by IL-8 as compared with GROα. When recombinant rabbit TNFα was incubated with synovial fluids obtained at 2 hours after IL-8 injection, the resultant TNFα activity was significantly decreased, an event that was completely restored by a serine protease inhibitor, phenylmethylsulphonyl fluoride (PMSF). Furthermore, TNFα activity was unveiled in the joints when IL-8 was intra-articularly injected with PMSF. These data suggest that TNFα is degraded by serine protease(s) in the case of IL-8. Taken together, the data clearly demonstrate the functional distinction between IL-8 and GROα, which may influence the inflammatory responses. (*Lab Invest 2002, 82:15–23*).

Neutrophil is the first cell population to migrate into inflammatory foci. Mediators regulating neutrophil recruitment have been extensively studied, and accumulating evidence suggests that the CXC chemokines play critical roles in this process (Baggiolini et al, 1995; Rollins, 1997). The CXC chemokines are a structurally related and functionally redundant family of proteins with specific leukocyte chemotactic activity that can be mainly divided into two subsets based on the presence or absence of specific amino acid residues, Glu-Leu-Arg (ELR) (Baggiolini et al, 1997; Zlotnik and Yoshie, 2000). The ELR CXC chemokines attract neutrophils, whereas non-ELR CXC chemokines are chemotactic for T lymphocytes and NK cells.

Interleukin-8 (IL-8: CXCL8) and growth-related oncogene α (GROα: CXCL1) are members of ELR CXC chemokines. These chemokines have been detected in a variety of clinical diseases that are characterized by a massive focal neutrophil infiltration, including acute respiratory distress syndrome, bacterial pneumonia, bacterial meningitis, and rheumatoid arthritis (Kunkel et al, 1995; Luster, 1998). We have recently shown that IL-8 and GROα are essential in the recruitment of neutrophils at the site of inflammation. Equivalent levels of IL-8 and GROα were detected in animal models of inflammation where neutralization of IL-8 or GROα with their specific antibodies resulted in decreased number of infiltrating neutrophils (Fukumoto et al, 1998; Matsukawa et al, 1998, 1999; Mo et al, 1999, 2000). The inhibition was further enhanced when both IL-8 and GROα were neutralized (Matsukawa et al, 1999). In vitro, IL-8 and GROα have been shown to be equally chemotactic for neutrophils (Balentien et al, 1990). Thus, it appears that IL-8 and GROα are equipotent neutrophil chemoattractants.

Questions remain about the relationship and the functional distinction between these CXC chemokines. To address these points, we injected either rabbit IL-8 or GROα into rabbit knee joints and analyzed the subsequent inflammatory responses in the joints. Our data suggest substantial differences between these closely related CXC chemokines, especially with respect to endogenously produced cytokines and their participation in the recruitment of neutrophils.

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Results

Kinetics of IL-8- or GRO-Induced Leukocyte Infiltration

Injection of either IL-8 or GROα into rabbit knee joints induced a massive neutrophil infiltration into the joints. In either case, the number of neutrophils peaked at 6 hours, decreased gradually, and subsided at 36 hours after the injection (Fig. 1). When compared at their peak (6 hours after the injection), the number of infiltrating neutrophils induced by IL-8 was more than that induced by GROα ($p < 0.05$). The number of mononuclear cells (>94% macrophages) induced by each chemokine was much less than that of neutrophils. The accumulation of mononuclear cells was first noted at 6 hours and peaked at 24 hours after the injection (Fig. 1).

Cytokine Production after Injection of IL-8 or GROα

To examine if IL-8 and GROα could induce other cytokines, levels of endogenously produced cytokines were measured after the injection of these chemokines. Intra-articular injection of IL-8 resulted in an increase in the synovial level of GROα. The GROα level peaked at 2 hours, decreased gradually, and then increased again at 12 hours after the injection (Fig. 2). Injection of GROα induced the production of IL-8, the level of which peaked at 2 and 12 hours after the injection (Fig. 2).

Tumor necrosis factor (TNF)α is regarded as the first cytokine-triggering inflammatory response (Driscoll et al, 1995). We next examined TNFα activity in the joints after the injection of either IL-8 or GROα. As shown in Figure 3A, no TNFα activity was detected after IL-8 injection. However, injection of GROα induced a significant level of TNFα activity in the joints. TNFα activity was measurable even at 1 hour, increased rapidly and peaked at 2 hours after the injection (Fig. 3A). When anti-TNFα mAb was coinjected with GROα, the GROα-induced IL-8 level at 12 hours, but not 2 hours, was inhibited by 50% (Fig. 3B). Thus, IL-8 and GROα are each capable of producing the other chemokine when they are injected into the joints. GROα, but not IL-8, induces TNFα activity, which in turn contributes to the production of IL-8 in a late phase. There were no detectable levels of IL-8, GROα, and TNFα in saline-treated controls (data not shown).

Neutrophil Infiltration after Neutralization of the Endogenous Cytokines

To investigate the involvement of endogenously produced cytokines in the neutrophil influx, anti-IL-8 IgG, anti-GROα mAb, or anti-TNFα mAb were injected together with IL-8 or GROα. IL-8- or GROα-induced neutrophil infiltration was completely blocked by the coadministration of anti-IL-8 IgG or anti-GROα mAb, respectively (not shown). The data in Figure 4A show that IL-8-induced neutrophil infiltration was not altered by the administration of anti-GROα mAb and/or anti-TNFα mAb at any time point examined. In contrast, GROα-induced neutrophil infiltration after 9 hours was significantly inhibited by the treatment with anti-IL-8 IgG, although there were no changes at 2 to 6 hours after the injection (Fig. 4B). When anti-TNFα mAb was injected with GROα, the neutrophil infiltration after 4 hours was significantly inhibited by 30% to 50%. Coadministration of anti-IL-8 IgG and anti-TNFα mAb further enhanced the inhibition (Fig. 4B). These data indicate that IL-8 acts directly on neutrophils, whereas GROα attracts neutrophils, in part, via the production of TNFα and IL-8. IL-8–induced GROα does not appear to be effective for attracting neutrophils.

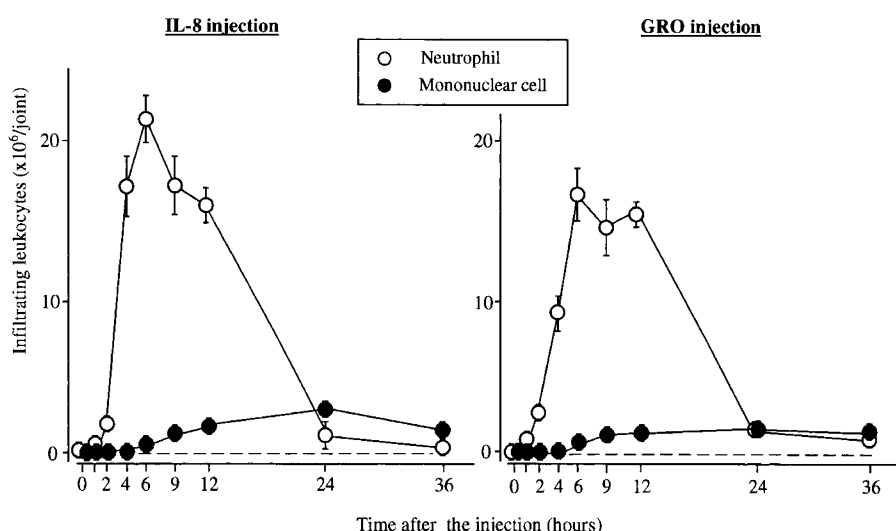

Figure 1.

Kinetics of IL-8- or GROα-induced leukocyte infiltration. At indicated intervals after intra-articular injection of IL-8 (left panel) or GROα (right panel) (10 μg each), the numbers of infiltrating neutrophils (○) and mononuclear cells (●) in the joints were recorded. Values represent the mean ± SE of 9–14 estimations from separate joints. The dotted line shows the background for vehicle-treated joints.

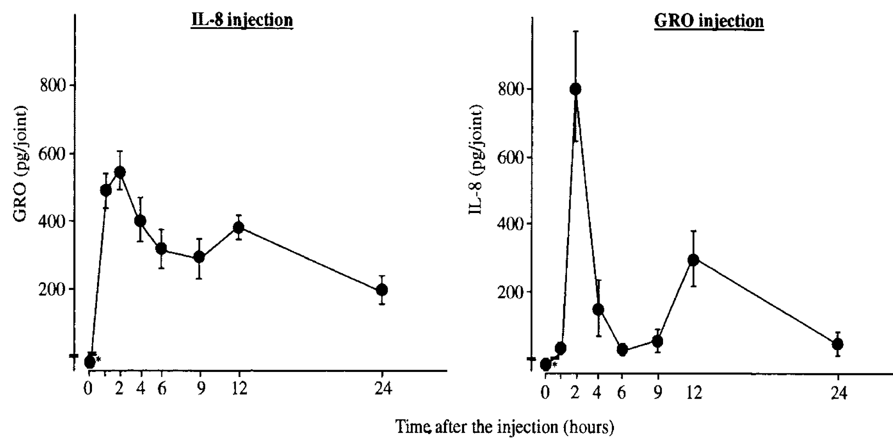

Figure 2.
Production of endogenous chemokines after the injection of either IL-8 or GROα. IL-8 or GROα (10 μg each) was injected into knee joints. At indicated intervals after the injection, synovial fluids were harvested and concentrations of endogenous GROα or IL-8 in the joints were measured, respectively. Values represent the mean ± SE of 9–14 estimations from separate joints. *Below detection level.

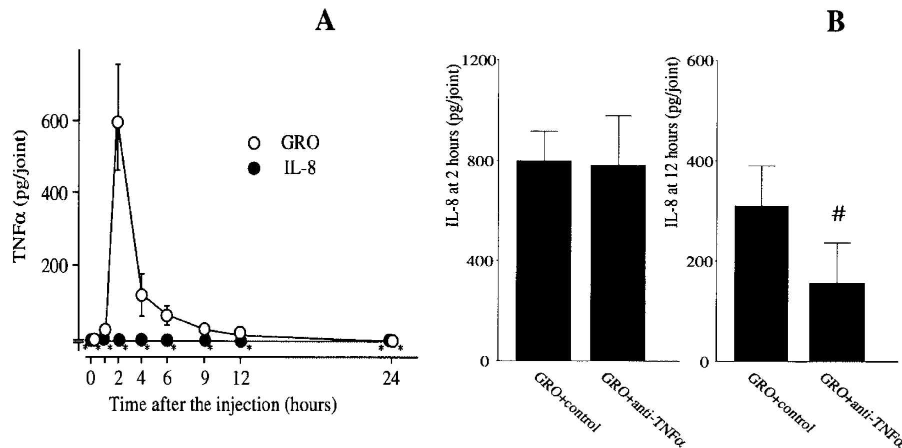

Figure 3.
TNFα activity after injection of either IL-8 or GROα. A, IL-8 or GROα (10 μg each) was injected into knee joints. At indicated intervals after the injection, synovial fluids were harvested and TNFα activity in the joints was measured. Values represent the mean ± SE of 9–14 estimations from separate joints. *Below detection level. B, GROα (10 μg) was injected into knee joints with anti-TNFα mAb (10 μg). Normal mouse IgG1 was used as a control. At 2 and 12 hours after the injection, synovial fluids were harvested and the concentration of IL-8 was measured. Values represent the mean ± SE of 8–10 estimations from separate joints. #$p < 0.05$, when compared with control.

mRNA Expression for TNFα by IL-8 and GROα

To further examine whether IL-8 is incapable of inducing TNFα, a cellular source of TNFα was next examined in vivo and the TNFα-producing capability by the cells was investigated in vitro. As shown in Figure 5A, TNFα activity in the joints after GROα injection was similar in normal and in neutrophil-depleted rabbits, suggesting that resident cells in the joints were responsible for TNFα production. Immunohistochemistry demonstrated that synovial cells were stained positively for TNFα (Fig. 5B). Therefore, synovial cells harvested from healthy rabbits were cultured and stimulated with either IL-8 or GROα.

The data shown in Figure 6 indicated that IL-8 failed to induce TNFα activity in cultured synovial cells even at a concentration as high as 1 μg/ml. On the other hand, GROα dose-dependently induced TNFα activity (Fig. 6). Heat-inactivated GROα (1 μg/ml, 100° C for 5 minutes) did not induce the activity (not shown). mRNA expression for TNFα in synovial cells was next examined by semiquantitated RT-PCR, which showed that IL-8 induced equivalent levels of the mRNA expression in synovial cells as compared with GROα (Fig. 7, A and B), despite the absence of TNFα activity in the culture supernatants. Thus, it is likely that TNFα may be inactivated in case of IL-8 stimulation.

Inactivation of TNFα by Serine Proteases

Many types of proteases exist in inflammatory foci that could inactivate TNFα. We next attempted to examine the possibility that coexisting proteases might inactivate TNFα in the joints. For this purpose, a known concentration of recombinant rabbit TNFα was added to synovial fluids obtained at 2 hours after IL-8 injection. The mixture was incubated for 1 hour at 37° C and the resultant TNFα activity was then assessed.

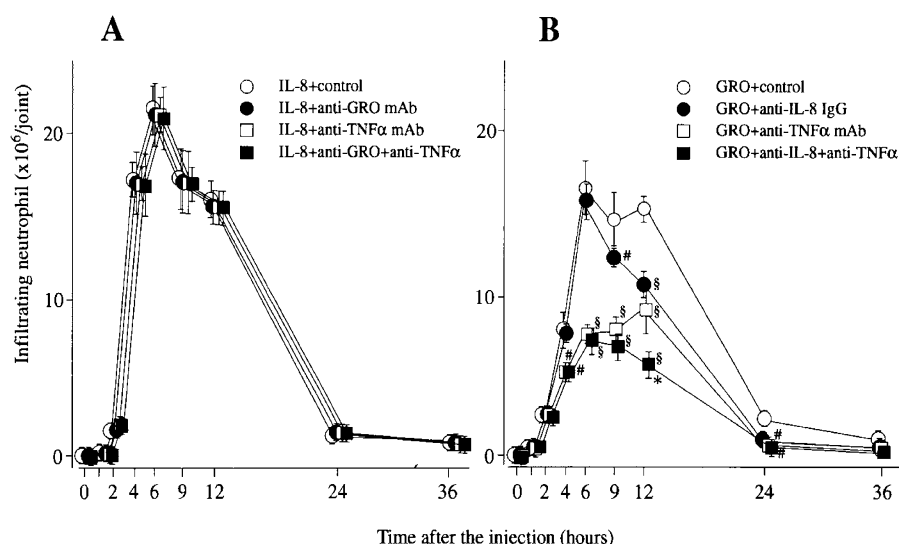

Figure 4.

Effects of anti-GROα mAb, anti-IL-8 IgG, and anti-TNFα mAb on IL-8- or GROα-induced neutrophil infiltration. Either IL-8 (A) or GROα (B) (10 μg each) was injected into knee joints with 10 μg of anti-TNFα mAb, anti-GROα mAb, or anti-IL-8 IgG, alone or in combination. At indicated intervals after the injection, the numbers of infiltrating neutrophils were counted. Values represent the mean $\pm$ SE of 8–12 estimations from separate joints. #$p < 0.05$, §$p < 0.01$, when compared with control. *$p < 0.01$, when compared with anti-IL-8 IgG or anti-TNFα mAb alone.

Phenylmethylsulphonyl fluoride (PMSF) alone did not affect the assay system (Fig. 8A, column 2). As a result, addition of synovial fluids significantly decreased TNFα activity in the mixture (Fig. 8A, column 4), as compared with control (Fig. 8A, column 3). When PMSF, a serine protease inhibitor, was added to the mixture, the diminished TNFα activity was completely restored to control level (Fig. 8A, column 5). These findings suggest that serine protease(s) existing in the synovial fluids after IL-8 injection may be responsible for TNFα inactivation. Experiments were further carried out by injecting PMSF into knee joints together with IL-8. Synovial fluids were harvested at 2 hours after the injection at a time when TNFα activity induced by GROα reached its peak (Fig. 3A). As shown in Figure 8B, injection of IL-8 or PMSF alone did not induce any appreciable level of TNFα activity. However, TNFα activity emerged when IL-8 was injected with PMSF. When PMSF was coinjected with GROα, there was no increase in the level of TNFα activity (not shown). These findings suggest that both IL-8 and GROα are capable of inducing TNFα, but the activity is abolished in case of IL-8 by serine protease(s).

Discussion

IL-8 and GROα are ELR CXC chemokines that have equivalent neutrophil chemotactic activities (Balentien et al, 1990). In the present study, we attempted to explore the functional difference between IL-8 and GROα in inflammation. Rabbits are a suitable animal to use to explore the role(s) of these chemokines in vivo, because rabbits, like humans, possess both IL-8 and GROα. These chemokines have not been identified in other experimental animals such as mice and rats (Jose et al, 1991; Yoshimura and Yuhki, 1991). Our results showed that IL-8 and GROα induced a massive neutrophil infiltration in joints. However, the number of infiltrating neutrophils induced by IL-8 was significantly higher than that induced by GROα, although the same molar concentrations of chemokines were injected, suggesting a potential difference(s) between these chemokines.

We here showed that each chemokine induced the production of the other chemokine when it was injected into the joints. Interestingly, IL-8–induced neutrophil infiltration was not inhibited by anti-GROα mAb despite the presence of GROα in the joints, whereas the GROα-induced response after 9 hours was significantly inhibited by anti-IL-8 IgG. These data indicate that endogenous GROα induced by IL-8 is not effective for the neutrophil influx, whereas GROα induces neutrophil infiltration in part via the action of IL-8. This conclusion appears to be reasonable, because IL-8 binds two CXC receptors, CXCR1 and CXCR2, whereas GROα binds only CXCR2 (Hall et al, 1999; Richardson et al, 2000; Wuyts et al, 1998). In the case of IL-8 injection, both CXCR1 and CXCR2 on neutrophils are likely to be already occupied by IL-8, providing no binding site for GROα. In the case of GROα injection, CXCR1 on neutrophils is expected to be vacant. Endogenous IL-8 can bind the receptor, enabling further neutrophil influx in a later phase of inflammation. Thus, the difference appears to be based on the distinct receptors corresponding to each chemokine.

The other interesting observation in the present study is that TNFα activity was not detected after IL-8 injection, whereas GROα induced a significant level of TNFα activity. Neutralization of TNFα did not affect neutrophil influx induced by IL-8, whereas GROα-induced neutrophil influx was significantly inhibited by the treatment. In vitro, GROα dose-dependently in-

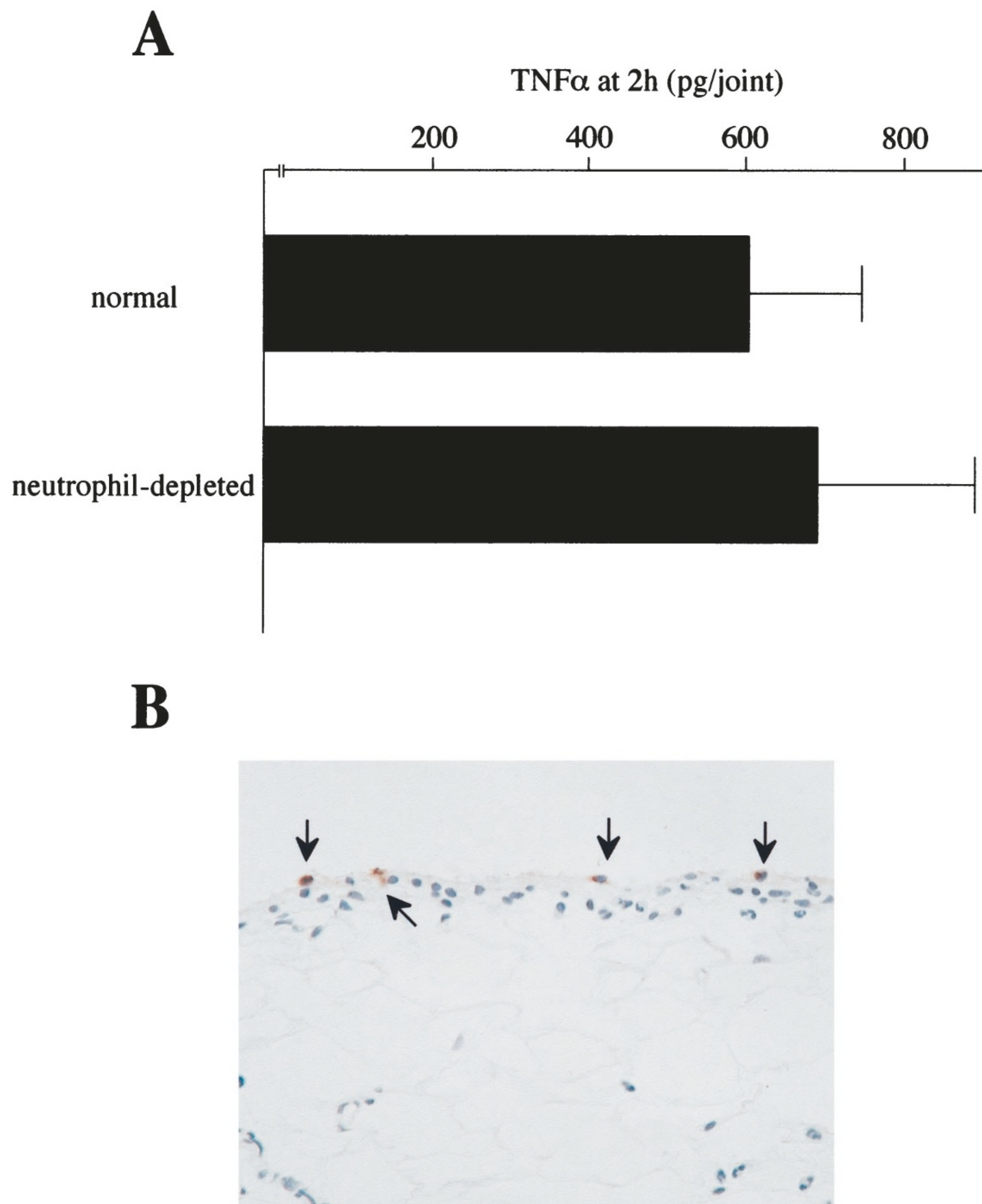

Figure 5.

Cellular source of TNFα after injection of GROα. A, GROα (10 μg) was injected into knee joints of normal or neutrophil-depleted rabbits. At 2 hours after the injection, synovial fluids were harvested and TNFα activity was measured. Values represent the mean $\pm$ SE of 9–14 estimations from separate joints. B, Synovial tissues obtained at 2 hours after GROα injection (10 μg) were stained with anti-TNFα IgG. Preimmune goat serum was used as a control. Original magnification $\times$ 1,000.

duced TNFα activity from synovial cells, the cells producing TNFα in joints after GROα injection, whereas IL-8 failed to induce the activity from the cells. When mRNA expression for TNFα was examined, synovial cells expressed equivalent levels of the mRNA after stimulation with either IL-8 or GROα. These data suggest that TNFα is likely to be inactivated in case of IL-8.

Previous reports have demonstrated that serine proteases that include trypsin, chymotrypsin, cathepsin G, and elastase degrade TNFα, resulting in a loss of TNFα bioactivity (Bauvois and Sanceau, 1992; Scuderi et al, 1991; van Kessel and van Strijp, 1991). The importance of CXCR1 in chemokine-mediated elastase release from neutrophils has also been reported (Jones et al, 1996). Thus, it is possible that IL-8, but not GROα, activates serine proteases, leading to an inactivation of TNFα. This appears to be a mechanism underlying the absence of TNFα activity after IL-8 injection, because synovial fluids obtained after IL-8 injection decreased exogenously added TNFα activity, and the activity was completely restored by PMSF. Likewise, TNFα activity was unveiled when IL-8 was injected with PMSF. Synovial cells are likely to be responsible for the source of proteases, because culture supernatants of synovial cells contained serine proteases that could inactivate TNFα. In addition, IL-8 failed to induce TNFα activity in neutrophil-depleted

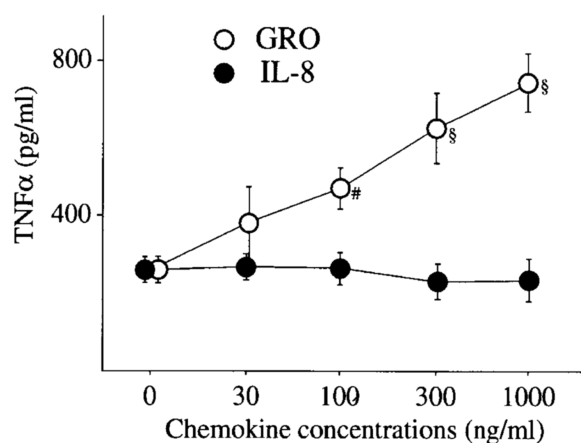

Figure 6.

TNFα activity in culture supernatants of synovial cells after the stimulation with IL-8 or GROα. Synovial cells harvested from knee joints of a healthy rabbit were stimulated with various concentrations of IL-8 or GROα. At 24 hours after the stimulation, culture supernatants were harvested and TNFα activity in the samples was measured. Values represent the mean ± SE of 8–10 estimations from culture supernatants. #$p < 0.05$, §$p < 0.01$, when compared with medium alone. Shown are representative data of three individual experiments.

rabbits (not shown). These results suggest that IL-8 is capable of inducing TNFα, but the activity is abolished by serine protease(s). The difference appears to be ascribable to the distinct receptors for IL-8 and GROα. Further study is required using antagonist/antibody against CXCR1 or specific CXCR1 agonist in this model. It should be noted that the restored TNFα activity by PMSF was not the same level as GROα, suggesting that other possibilities may exist. There may be a change in the transcription of TNFα. An up-regulation of TNFα receptor and soluble TNFα receptor may be responsible for the difference.

In conclusion, there appear to be functional differences between IL-8 and GROα in inflammation. Both chemokines induced a massive neutrophil influx, but the IL-8–induced response was more than that induced by GROα. IL-8 directly attracts neutrophils, whereas GROα attracts indirectly, in part via IL-8 and TNFα. TNFα activity was diminished in case of IL-8 injection due to serine protease(s). This is the first report showing in vivo functional distinction between closely similar CXC chemokines, IL-8 and GROα, which may influence the inflammatory responses.

Materials and Methods

Cytokines and Their Antibodies

Recombinant rabbit IL-8, GROα, and TNFα were purified, as described elsewhere (Matsukawa et al, 1995, 1997, 1999). Neutralizing anti-rabbit IL-8 IgG and anti-rabbit GROα IgG were raised in a goat, and purified with affinity column coupled with each chemokine (Matsukawa et al, 1997, 1999). Neutralizing anti-rabbit TNFα mAb was kindly provided by Dr. H. Nariuchi (The University of Tokyo, Japan) (Haranaka et al, 1985). Neutralizing anti-rabbit GROα mAb (IgG1) was developed as described previously (Matsukawa

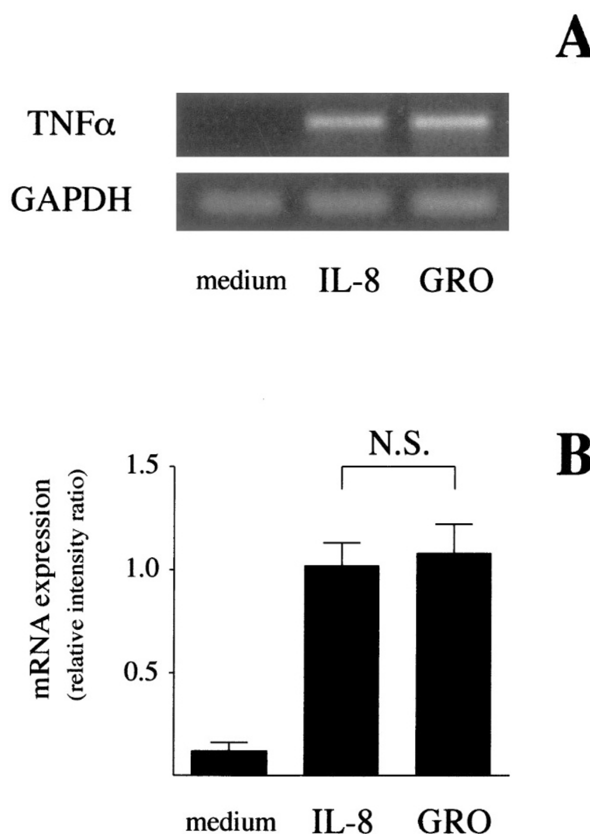

Figure 7.

TNFα mRNA expression in synovial cells after the stimulation with IL-8 or GROα. A, Synovial cells harvested from knee joints of a healthy rabbit were stimulated with 1 μg/ml IL-8 or GROα for 1 hour at 37° C. RNA was extracted from the cells and mRNA expression for TNFα was examined by RT-PCR. Expression of GAPDH was used as a control. Shown are representative photographs of three individual experiments. B, Ethidium bromide stained PCR products were photographed, and then the images were digitized and analyzed. Results are expressed as a ratio of each PCR product/GAPDH band density. Values represent the mean ± SE of six different estimations of PCR data. PCR was performed in duplicate for each experiment.

et al, 1999). Polyclonal antibodies and mAb used in this study did not cross-react with other rabbit cytokines available (not shown). Endotoxin contamination in these reagents was removed by Kurimover II (Kurita Kogyo Co., Tokyo). Endotoxin contents in the resultant samples were less than 0.02 ng/mg protein (QCL-1000; Daiichi Pure Chemicals, Tokyo, Japan).

Induction of Arthritis

Female New Zealand white rabbits (weight 2.2 to 2.5 kg) were anesthetized by giving pentobarbital sodium (30 mg/kg) intravenously, and then either IL-8 or GROα (10 μg each in 500 μl of endotoxin-free saline) was injected into the knee joints through the suprapatellar ligament in the presence of 10 U/ml polymyxin B (Pfizer Pharmaceutical Co., Tokyo, Japan). At various intervals after the injection, rabbit were anesthetized, bled, and killed. The knee joints were washed with 1 ml saline and the joint fluids were harvested and centrifuged at 6,000*g* for 1 minute at 4° C. The cell-free synovial fluids were harvested and stored at

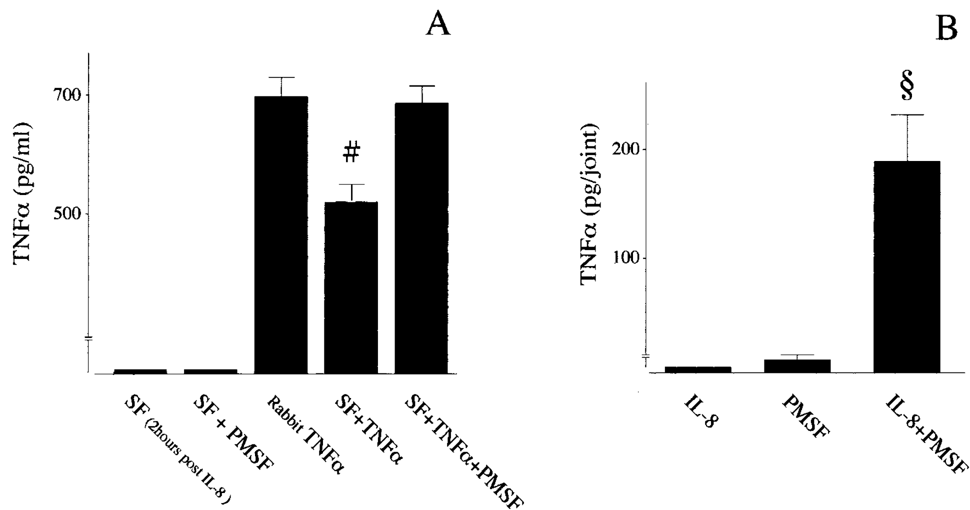

Figure 8.

Inactivation of TNFα by serine proteases. A, Rabbit TNFα was added to synovial fluids (*SF*) obtained at 2 hours after the injection of IL-8, and the mixture was incubated for 1 hour at 37° C in the presence or absence of phenylmethylsulphonyl fluoride (PMSF; final concentration, TNFα, 1 ng/ml; PMSF, 0.1 mM). TNFα activity in the cultured samples was then measured. Values represent the mean ± SE of 8–10 estimations from culture supernatants. #$p < 0.05$, when compared with rabbit TNFα alone (column 3). Shown are representatives of three individual experiments. B, IL-8 (10 μg) was injected into knee joints with or without 1 mM PMSF. At 2 hours after the injection, synovial fluids were harvested and TNFα activity was measured. Values represent the mean ± SE of 9–14 estimations from separate joints. §$p < 0.01$, when compared with IL-8 or PMSF alone.

−80° C until analysis. Cell pellets were resuspended in saline and the numbers were counted with hemocytometer. Differential cell analysis was made with Wright-Giemsa staining. The Animal Care Committee of Kumamoto University School of Medicine approved the animal experiments.

In Vivo Experimental Protocol

To neutralize GROα, IL-8, or TNFα activity in knee joints, 10 μg of anti- GROα mAb, anti-IL-8 IgG, or anti-TNFα mAb was injected into knee joints, respectively, together with IL-8 or GROα in a total volume of 500 μl. Ten micrograms were regarded as sufficient, because higher doses (30 to 100 μg) did not further reduce leukocyte infiltration (not shown). Ten micrograms of mouse IgG1 (Sigma Chemical, St. Louis, Missouri) and preimmune goat IgG were used as controls, which did not induce leukocyte infiltration and cytokine production (not shown). In different experiments, 1 mM of PMSF (Sigma) was injected into knee joints together with IL-8 or GROα (total 500 μl). These reagents were randomly chosen and injected into bilateral knee joints. Injection of reagents into one side of the knee joints did not affect the leukocyte infiltration and cytokine production induced in the contralateral knee joints. At various intervals after the injection, rabbits were anesthetized, bled, and killed. Samples were harvested and treated as described above.

Measurement of Cytokines

IL-8 and GROα were quantitated by ELISA and time-resolved fluoroimmunoassay, respectively, as described previously (Matsukawa et al, 1997, 1999). The detection limit was 30 and 3 pg/ml, respectively. TNFα level was determined by L929 cell cytotoxic assay, as previously described (Flick and Gifford, 1984). Concentrations of TNFα were determined from known concentrations of recombinant TNFα used in the same assay. The specificity was confirmed by preincubating samples with neutralizing anti-TNFα mAb. The detection limit was 30 pg/ml. Synovial fluids were digested with 10 U/ml hyaluronidase (Sigma) for 1 hour at 37° C, before assays.

Preparation of Neutrophil-Depleted Rabbits

Neutrophil-depleted rabbits were prepared by intravenous injection of 0.75 mg/kg vinblastine (Sigma) 3 days before the experiments (Rosenshein et al, 1979). The number of peripheral leukocytes before and after vinblastine treatment were 12.7 ± 0.7 × 10^6/ml and 3.4 ± 0.8 × 10^6/ml, respectively. The majority of leukocytes in neutrophil-depleted rabbits were mononuclear cells, whereas 30% to 50% of the leukocytes in normal rabbits were neutrophils.

Immunohistochemistry

Freshly isolated synovial tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and 4-μm-thick sections were made. After blocking endogenous peroxidase with 0.3% H_2O_2 in methanol, the tissue sections were treated with 5% normal rabbit serum and incubated overnight at 4° C with goat polyclonal anti-rabbit TNFα IgG. Preimmune goat IgG was used as a control. The sections were then incubated for 30 minutes with 5 μg/ml biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, California), rinsed, and incubated for 30 minutes with avidin-biotin-peroxidase complex (Vector). As a chromogen, diaminobenzidine (DAB; Wako Chemical In-

dustries, Osaka, Japan) was used. Counter-staining was performed with hematoxylin.

Cell Culture

Synovial tissues were harvested from bilateral knee joints of healthy rabbits, minced, and digested with collagenase (4 mg/ml; Worthington Biochemical, Freehold, New Jersey) for 30 minutes at 37° C. The cell suspension was washed, resuspended in DMEM supplemented with 10% FBS and antibiotics, and plated in 24-well culture plates. One hour later, medium was changed and adherent cells were then cultured for 4 to 6 days. Upon confluence, cells were stimulated with various concentrations of IL-8 or GROα, and the cell-free culture supernatants were harvested at appropriate intervals after the stimulation. Total RNA in adherent synovial cells was extracted with RNeasy Mini Kit (QIAGEN Co., Tokyo, Japan).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using the ThermoScript RT-PCR System (GIBCO-BRL, Gaithersburg, Maryland), according to the manufacturer's instructions. The primers were designed to amplify rabbit TNFα and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) referred to the cDNA sequence. The primers were as follows: TNFα, sense primer: 5'-TCAGCTTCTCGGGCCCTGAGTGAC-3'; anti-sense primer: 5'-TCACAGGGCAATGATCCCAAAGTAGAC-3'. GAPDH, sense primer: 5'-GATCCATTCATTGACCTCC-3'; anti-sense primer: 5'-GATCTCGCTCCTGGAAGATG-3'. PCR was carried out at 35 cycles of 94° C for 1 minute, 55° C for 1 minute, and 72° C for 2 minutes. Ten microliters of PCR products were subjected to electrophoresis on a 2% agarose gel in the presence of ethidium bromide, photographed, digitized, and the band densities were measured with NIH image. Results are expressed as a ratio of each PCR product/GAPDH band density; this represents semiquantitative analysis.

Culture of Synovial Fluids with Reagents

Rabbit TNFα (1 μl of 100 ng/ml) was added to synovial fluids (100 μl) obtained at 2 hours after the injection of IL-8, and the mixture was incubated for 1 hour at 37° C in the presence or absence of PMSF (final concentration, TNFα, 1 ng/ml; PMSF, 0.1 mM). TNFα activity in the cultured samples was then measured. The dose of PMSF was predetermined, based on the maximum effects achievable. PMSF at this dose did not affect the TNFα assay.

Statistics

Statistical significance was evaluated by two-tailed unpaired Student's *t* test. $P < 0.05$ was regarded as statistically significant. All data were expressed as mean ± SE.

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