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Expression and Production of the CXC Chemokine Growth-Related Oncogene-α by Human Eosinophils[1]

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Eosinophils are seen together with neutrophils at sites of inflammation. However, their roles are not clear. In addition, eosinophils infiltrate tumor tissue in some neoplastic diseases. In this study, we show that large amounts of the neutrophil-activating CXC chemokine growth-related oncogene (GRO)-α can be produced by human eosinophils. Eosinophils showed presence of preformed GRO-α in the crystalloid-containing specific granules (190 pg/2 $\times$ 10^6 cells). During incubation, a strong increase in *GRO-α* gene expression was seen. At a low cell density, addition of TNF-α or IL-1β increased the production of GRO-α in eosinophils, which was not the case at a higher cell density. Eosinophils can produce TNF-α themselves, and neutralizing Abs against TNF-α significantly inhibited GRO-α production. This suggests that autocrine and paracrine effects from TNF-α can be important when up-regulating *GRO-α* gene expression. In contrast, IFN-γ, a prototypic Th1-cytokine, down-regulated expression of GRO-α. This may be important during resolution of inflammation but also suggests different roles for eosinophils depending on the inflammatory context. Tumor-infiltrating eosinophils in Hodgkin's disease of the nodular sclerosing type are associated with a poor prognosis. Eosinophils from such tumor tissue showed an abundant expression of GRO-α. The GRO-α receptor CXCR2 was also detected in tumor tissue, proposing interactions between eosinophils and the tumor. Our findings suggest that eosinophils can promote inflammation through recruitment of CXCR2-bearing cells. In addition, this feature of the eosinophils indicates a role for these cells in the biology of certain tumors. ***The Journal of Immunology*, 2003, 170: 5309–5316.**

Eosinophils are a typical component of the inflammatory response seen in diseases such as allergic asthma, ulcerative colitis, helminthic infestation, and some neoplastic diseases, e.g., Hodgkin's disease (1). In contrast to neutrophils, also seen at sites of inflammation, eosinophils may have a long life span in tissues and can produce several cytokines and chemokines. Therefore, eosinophils are likely to have regulatory functions during inflammation (2).

Chemokines are proinflammatory peptides that can activate leukocytes by binding to G protein-coupled receptors on the cell surface. They can be divided into C, CC, CXC, and $C(X)_3C$ chemokines based on the arrangement of conserved cysteine residues. The CXC chemokines can be further divided into ELR^+ and ELR^- based on the presence or absence of the sequence motif glutamic acid-leucine-arginine (ELR) (3). Growth-related oncogene (GRO)[3]-α and IL-8 are both ELR^+ CXC chemokines. GRO-α possesses potent neutrophil-stimulating activity by inducing chemotaxis, shape charge, a rise in intracellular free calcium levels, exocytosis, and the respiratory burst in neutrophils (4). Initially, GRO-α was isolated and characterized by its growth stimulatory activity on malignant melanoma cells (5). In addition, this peptide can regulate endothelial cell proliferation, stimulating angiogenesis (6).

In this study, we show that eosinophils express the CXC chemokine GRO-α. This feature of eosinophils may promote inflammation at tissue sites where eosinophils accumulate. It can also be important for the interaction between eosinophils and tumor cells.

Materials and Methods

Special reagents

Recombinant human IL-1β, IL-5, IFN -γ, and TNF-α were from R&D Systems (Abingdon, U.K.). mAbs against GRO-α, TNF-α, CXCR2, and irrelevant mouse monoclonal IgG1, and ELISA kits for the measurement of GRO-α, IL-8, and TNF-α were purchased from R&D Systems. The mAb EG1 against eosinophil cationic protein (ECP) was from Pharmacia (Uppsala, Sweden).

Isolation of eosinophils

Citrated blood was obtained from healthy volunteers after informed consent, and eosinophils were isolated essentially as described (7). In short, after isolation of granulocytes on Ficoll-Paque (Pharmacia), immunomagnetic beads coated with Abs to CD16 (Miltenyi Biotec, Gladbach, Germany) were used to retrieve the neutrophils in a magnetic column, allowing the isolation of highly purified eosinophils. The purity of eosinophils was >98%; contaminating cells were neutrophils and lymphocytes, as judged by routine May-Grünwald-Giemsa staining. Trypan blue exclusion showed >99% cell viability.

Immunocytochemistry

Purified eosinophils were fixed in paraformaldehyde (4%; v/v) in PBS, dehydrated, and embedded in paraffin. Immunohistochemistry was performed essentially as described (8). In short, sections (3 μm in thickness) were cut, and after deparaffinization and rehydration, they were incubated with a mAb against GRO-α or an isotype-matched irrelevant Ab. This was followed by incubation with a secondary ALP-conjugated goat anti-mouse Ab (DAKO, Glostrup, Denmark). To visualize bound Abs, the sections were developed through exposure to a mixture containing naphthol phosphate, fast red, and levamisol (all from Sigma-Aldrich, St. Louis, MO). The sections were counterstained with Mayer's hematoxylin.

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[3] Abbreviations used in this paper: GRO, growth-related oncogene; ECP, eosinophil cationic protein; MIP, macrophage-inflammatory protein.

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Immunoelectron microscopy

Purified eosinophils were fixed for 24 h in 4% paraformaldehyde in PHEM buffer (120 mM PIPES, 50 mM HEPES, 8 mM $MgCl_2$, 40 mM EGTA; pH 6.9) and then processed for ultrathin cryosectioning as previously described (9). Forty-five-nanometer cryosections were cut at −125°C using diamond knives (Drukker, Cuijk, The Netherlands) in an ultracryomicrotome (Leica, Vienna, Austria) and transferred with a mixture of sucrose and methylcellulose onto formvar-coated copper grids (10). The grids were placed on 35-mm petri dishes containing 2% gelatin. Cryosections were incubated with a specific mAb against human GRO-α (R&D Systems), followed by incubation with rabbit anti-mouse IgG and then incubated with 10-nm protein A-conjugated colloidal gold (Electron Microscopy Laboratory, Utrecht University, Utrecht, The Netherlands). After immunolabeling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a CM 10 electron microscope (Philips, Eindhoven, The Netherlands). For control, an irrelevant isotype-matched mouse mAb replaced the primary Ab.

Measurement of GRO-α and IL-8 in cell homogenates and in medium

Eosinophil cell lysates were obtained by lysing 4×10^6 cells/ml in 1% Triton X-100 (v/v) in PBS supplemented with 10 mM benzamidine for 20 min on ice. The lysate was centrifuged for 20 min at $21{,}000 \times g$ to clear the supernatant from particulate cellular debris. The supernatants were stored at −70°C until analyzed by ELISA. During prolonged incubation, eosinophils (0.5 or 2×10^6/ml) were suspended in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated FCS and gentamicin (10 μg/ml) in 24- or 96-well plates (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere containing 5% CO_2. In selected experiments, IL-1β (0.1, 1, 5, and 10 ng/ml), IL-5 (1 nM), TNF-α (0.1, 1, 5, and 10 ng/ml), IFN-γ (1, 10, 100, and 500 U/ml), or medium alone were added to the cells, and coincubation was performed for 24 h; thereafter, the plates were centrifuged for 10 min at $500 \times g$, the supernatants were collected, and the cells were lysed as described above. The cell viability was >99% immediately after isolation, and >98% after 16 h, >96% after 24 h, and >90% after 48 h of incubation as judged by trypan blue exclusion. The supernatants and cell lysates were stored at −70°C until analyzed by ELISA.

Measurement of GRO-α in cell lysates and in supernatants

The GRO-α, TNF-α, and IL-8 contents were measured by ELISA. The measurements were performed in duplicate. According to the manufacturer, the GRO-α ELISA does not recognize related peptides such as epithelial neutrophil-activating protein 78, IL-8, GRO-β, GRO-γ, monocyte chemoattractant protein-1, macrophage-inflammatory protein (MIP)-1α, MIP-1β, or RANTES. Neither does the IL-8 ELISA recognize related peptides. The ranges were 15–2000 pg/ml (GRO-α), 15–2000 pg/ml (IL-8), and 4–1000 pg/ml (TNF-α).

In situ hybridization

Detection of *GRO-α* gene expression on a single-cell level was performed on cytospin preparations of eosinophils that had been incubated for 16 h in medium as described above. A digoxygenin-labeled oligonucleotide with the sequence 5′-CCC GCG CTG CTC TCT CCG CCG CCC CCA GC-3′ was used to detect GRO-α expression. An oligonucleotide recognizing transcripts of prostate-specific Ag with the sequence 5′-GA GGG TGA ACT TGC GCA CAC ACG TCA TTG-3′ served as a negative control. In situ hybridization was performed as previously described (11). In short, slides were hybridized with probe (200 ng/ml) overnight at 30°C and thereafter washed in highly stringent conditions. To detect hybridization signals, the sections were incubated with alkaline phosphatase-conjugated Fab derived from anti-digoxigenin IgG (Roche, Basel, Switzerland). After thorough washing, sections were developed by exposure to nitroblue tetrazolium chloride and levamisol. For control purposes, adjacent sections were hybridized with digoxigenin-labeled oligo(dT) to ensure successful fixation and hybridization.

Detection of GRO-α and IL-8 expression by RT-PCR

Total cellular RNA was isolated using a kit based on a modified single-step procedure by acid guandinium thiocyanate-phenol-chloroform extraction (Total RNA Isolation kit; BD Biosciences, Stockholm, Sweden).

The primer sequences for detection of GRO-α and IL-8 expression by RT-PCR were chosen to be intron-spanning to exclude amplification of genomic DNA. The primer sequences for GRO-α were 5′-TGG CCA TTT GCT TGG ATC CGC CAG CCT-3′ (sense) and 5′-TAG CCA CAC TCA AGA ATG GGC GGA AAG CTT-3′ (antisense), and for IL-8, 5′-ATG ACT TCC AAG CTG GCC GTC-3′ (sense) and 5′-GGA GTA TGT CTT TAT GCA CTG ACA TCT-3′ (antisense). The housekeeping gene *GAPDH* was used as a control. The *GAPDH* primers were 5′-ACC ACC ATG GAG AAG GCT GG-3′ (sense) and 5′-CAC AGT GTA GCC CAG GAT GC-3′ (antisense).

The RT-PCR were performed using premixed, predispensed reaction tubes to which 100 ng of RNA and primer pairs were added (Ready-To-Go RT-PCR Beads; Pharmacia). First-strand cDNA synthesis was performed at 39°C for 45 min, followed by denaturation at 95°C for 5 min. The conditions for the PCR were as follows: denaturation at 94°C for 60 s, annealing at 53°C for 90 s, and elongation at 72°C for 120 s, for 40 cycles. Samples (10 μl) of the PCR mixtures were loaded on a 2% agarose gel and stained with ethidium bromide. The PCR products were detected by UV light in a computer-based gel documentation system (Gel Doc 2000; Bio-Rad, Hercules, CA).

Allergic and nonallergic blood donors

Blood was drawn by venipuncture from both healthy donors and donors suffering from seasonal allergic rhinoconjunctivitis during the grass and tree pollen season in Sweden after approval by the ethical committee at Lund University (no. LU 724-01) and informed consent from the donors. Healthy donors were characterized by absence of allergic history, measurement of total IgE, and lack of specific IgE Abs against a panel of 12 different airway Ags (Phadiatope; Pharmacia). Allergics were characterized by a history of seasonal rhinoconjunctivitis and ongoing symptoms, increased concentrations of total IgE, and the presence of specific IgE Abs against grass- or tree pollen (RAST; Pharmacia). None of the allergics were on current medication.

Immunohistochemistry and in situ hybridization on tumor tissue

Archived diagnostic biopsies obtained at presentation of disease in patients suffering from Hodgkin's disease of the nodular sclerosing type was used to detect GRO-α containing cells using the method described for immunocytochemistry above. In the case of detecting CXCR-2-bearing cells, a detection kit was used (DAKO ChemMate Detection kit; peroxidase/DAB, rabbit/mouse; Bio-Tek Solutions, Winooski, VT) and a staining machine (DAKO TechMate 500/1000 instrument; Bio-Tek Solutions). Briefly, thin sections were deparaffinized, rehydrated, and incubated with mAbs against GRO-α, CXCR2, or an irrelevant isotype-matched Ab. After extensive washings, bound Abs were detected by biotinylated goat anti-mouse IgG. The immunoreactivity was visualized using the manufacturer's protocol for the peroxidase/3,3′-diaminobenzidine tetrahydrochloride reagent in the ChemMate kit (Bio-Tek Solutions). The sections were counterstained with Mayer's hematoxylin solution.

In situ hybridization was performed as described above for cytospin preparations of eosinophils. To confirm the eosinophil phenotype of GRO-α-expressing cells, the sections that had been subject to in situ hybridization were incubated with a mAb against the eosinophil granule protein ECP (EG1; Pharmacia) followed by detection of bound Ab by secondary FITC-conjugated goat anti-mouse Abs (DAKO).

Statistics

Student's *t* test for paired data was used for statistical calculations. Statistical significance was set at $p \leq 0.05$ and $p \leq 0.01$.

Results

Detection of GRO-α in eosinophils

Sections of freshly isolated eosinophils showed immunologically detectable GRO-α appearing as a coarse granular staining pattern of the cytoplasm (Fig. 1*a*). Replacement of the specific primary Ab with an irrelevant isotype-matched Ab resulted in loss of staining (Fig. 1*b*).

Immunogold electron microscopy was used to determine the subcellular localization of GRO-α in eosinophils. Abundant labeling, showing the presence of GRO-α, was seen in the crystalloid-containing specific granules (Fig. 1*c*). No labeling was found in control sections where the primary Ab was replaced by an isotype-matched irrelevant Ab (not shown).

In lysates of highly purified eosinophils, 190 ± 38 pg (mean ± SEM; range, 86–284 pg; $n = 5$) of GRO-α was detected per 2×10^6 cells.

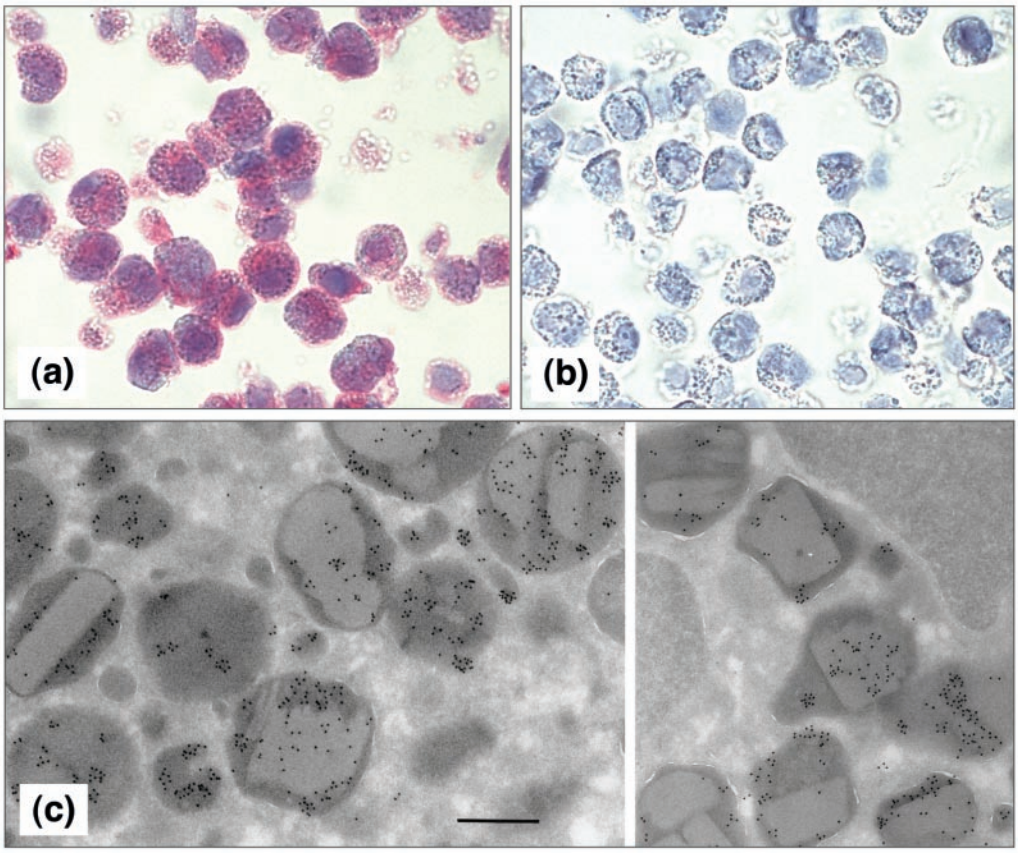

FIGURE 1. Detection of GRO-α in eosinophils by immunocytochemistry and immunoelectron microscopy. *a*, Immunocytochemical detection of GRO-α in eosinophils. Sections of purified eosinophils were incubated with a mAb against GRO-α, and bound Ab was visualized with a secondary goat anti-mouse Ab conjugated with alkaline phosphatase. An enzymatic reaction resulted in red staining. A granular staining pattern is seen in all cells. *b*, Replacement of the primary specific Ab by an isotype-matched irrelevant Ab resulted in loss of staining. *c*, GRO-α is present in crystalloid-containing granules of eosinophils. Presence of GRO-α is visualized by immunoelectron microscopy using specific Abs and colloidal gold (10-nm gold particles). Bar, 500 nm.

GRO-α expression by eosinophils during prolonged incubation

Gene expression of GRO-α in eosinophils was analyzed by semi-quantitative RT-PCR immediately after isolation and after 4, 8, and 16 h of incubation. In freshly isolated eosinophils, a weak constitutive expression was seen, and after 4 h of incubation, a strong increase in GRO-α expression was observed, remaining high during the observation time (Fig. 2*a*).

Increasing concentrations of GRO-α were detected with time in the medium during prolonged incubation (Fig. 2*b*). The amounts released to the medium and present in cell lysates after 0, 16, 24, and 48 h of incubation were measured using ELISA. The amounts detected in the medium exceeded the amounts detected in cell lysates, showing that GRO-α is mainly synthesized de novo.

GRO-α gene expression on a single-cell level was detected by in situ hybridization. Eosinophils that had been incubated for 16 h showed a homogeneous expression of GRO-α (Fig. 2*c*). Incubation with an irrelevant probe served as a negative control (Fig. 2*d*).

GRO-α and IL-8 expression are concomitantly up-regulated in eosinophils and the degree of expression is not dependent on the presence of donor allergy

The constitutive, as well as the stimulated expression after 16 h of incubation, were compared for GRO-α and the related CXC chemokine IL-8 by RT-PCR. A large variation in gene expression between different donors was observed. In general, IL-8 showed a higher constitutive expression than GRO-α. After 16 h of incubation, both genes were up-regulated, although IL-8 was to a higher degree than GRO-α (Fig. 3*a*).

To investigate a possible correlation between the amounts of produced GRO-α and IL-8, eosinophils were incubated in medium for 24 h. The released amount of GRO-α and IL-8 were measured by ELISA. A strong correlation between the two was observed (Fig. 3*b*).

The heterogeneity with respect to constitutive GRO-α expression in eosinophils could be explained by the presence of donor allergy, because increased constitutive expression of IL-8 was suggested in allergic asthmatics (12). Blood was obtained from donors with confirmed ongoing seasonal rhinoconjunctivitis during pollen season and donors without a history of allergy. A large variation in both GRO-α and IL-8 synthesis was observed between donors. However, no differences were observed comparing allergic and nonallergic donors for neither GRO-α nor IL-8 synthesis (Fig. 3*c*).

GRO-α synthesis by eosinophils is dependent on autocrine and paracrine effects from TNF-α

It has been shown that GRO-α expression can be induced by TNF-α (13). Therefore, we investigated whether TNF-α produced by eosinophils themselves were of importance. Eosinophils were incubated in the presence of a neutralizing Ab against TNF-α or an isotype-matched control Ab for 24 h. The amount of GRO-α released by the eosinophils significantly decreased in the presence of neutralizing Abs (Fig. 4*a*). Low amounts of TNF-α were detected

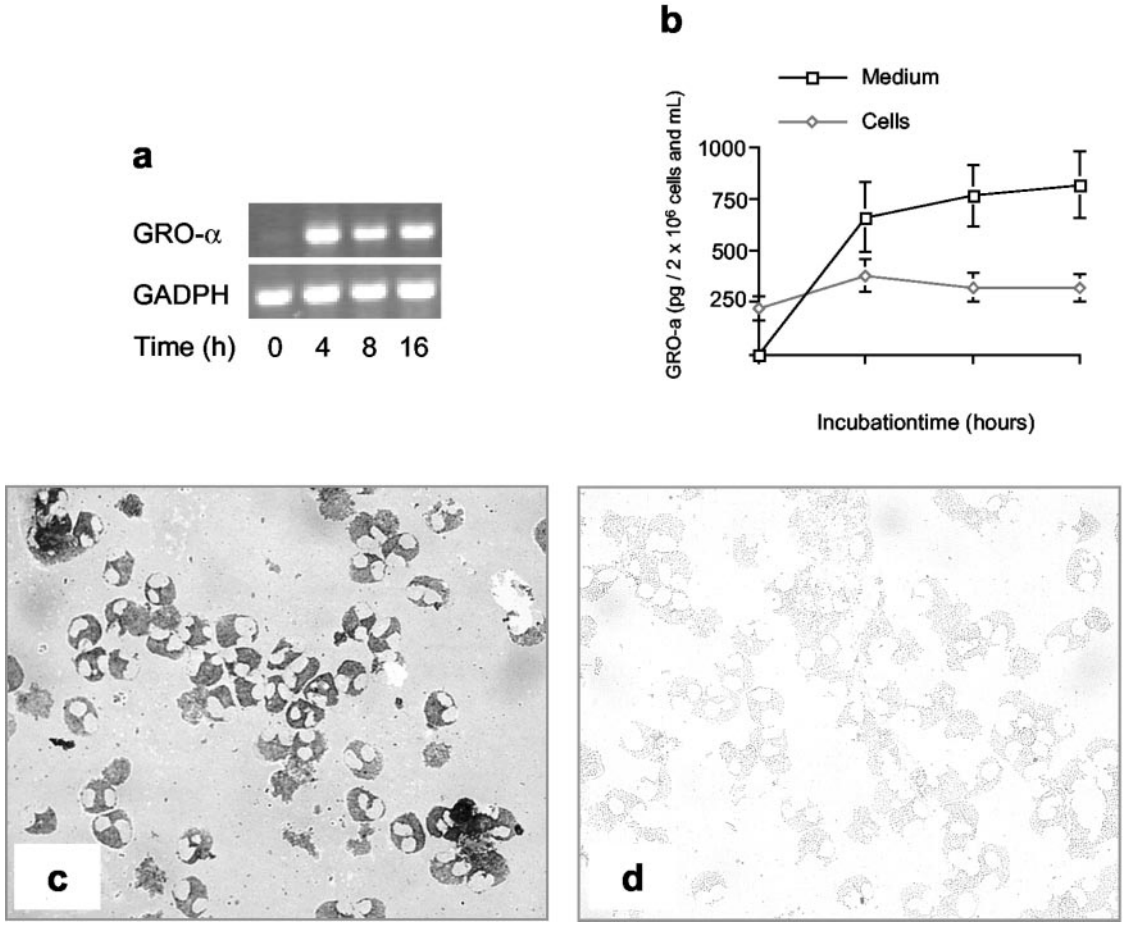

FIGURE 2. GRO-α expression during prolonged incubation in vitro. *a*, Increased *GRO-α* gene expression during prolonged incubation as detected by RT-PCR. Eosinophils (2×10^6/ml) were incubated in medium, and RNA was isolated at the indicated time points and thereafter was subjected to RT-PCR. A strong increase in *GRO-α* gene expression was seen within 4 h of incubation. The house-keeping gene *GADPH* is shown to ensure equal loading of template. The kinetics from one representative of four donors is shown. *b*, Release of GRO-α peptide during prolonged incubation measured by ELISA. Eosinophils (2×10^6/ml) were incubated in medium. At the indicated time points, cells and medium were collected, and the GRO-α content was determined. The data shown represent mean ± SEM from five separate experiments. *c*, Expression of GRO-α in eosinophils was detected by in situ hybridization. Cytospin preparations of eosinophils were prepared after 16 h of incubation and subjected to in situ hybridization. A digoxygenin-labeled antisense oligonucleotide specific for GRO-α was used. Hybridization was detected by an alkaline phosphatase reaction product, which resulted in a blue-violet staining of the cytoplasm of the cells. A homogeneous expression of the *GRO-α* gene is seen. *d*, Incubation of eosinophils with an antisense oligonucleotide against prostate-specific Ag, serving as a negative control, results in loss of staining.

in the medium (data not shown). In addition, eosinophils were incubated at two different densities and stimulated with IL-1β, TNF-α, or both cytokines. At the lower cell concentration (0.5×10^6 cells/ml), both TNF-α and IL-1β increased the GRO-α production. However, no additive or synergistic effects were detected. At the higher cell concentration (2×10^6 cells/ml), the effect from the added cytokines was weak or absent, suggesting that the system was saturated with respect to autocrine and paracrine effects from TNF-α (Fig. 4*b*). Dose-response experiments with TNF-α and IL-1β were also performed, at the lower cell density, showing an increased synthesis of GRO-α with increased concentrations of the cytokines (Fig. 4, *c* and *d*).

Effects of IFN-γ and IL-5 on GRO-α production

The gene expression of GRO-α decreased in the presence of IFN-γ as detected by RT-PCR. There was a variation between donors as shown in Fig. 5*a*. Incubation of eosinophils for 24 h in medium containing IFN-γ caused a significant decrease in GRO-α synthesis (Fig. 5*b*). This is similar to the effect of IFN-γ on GRO-α synthesis in neutrophils (14). To see whether the effect from IFN-γ was dose-dependent, cells where incubated in medium for 24 h with increasing concentrations of IFN-γ. A higher dose of IFN-γ resulted in a stronger inhibitory effect (Fig. 5*c*). In addition, the effects of IL-5, an eosinophil-activating cytokine, were investigated. Eosinophils, isolated from 33 different donors, both healthy persons and individuals with ongoing allergy, were incubated in medium alone or in the presence of IL-5 (1 nM). Addition of IL-5 showed both stimulatory and inhibitory effect on the production of GRO-α. The effect was not dependent on presence of allergic disease (data not shown). In Fig. 6, data obtained from six representative donors are shown.

GRO-α in Hodgkin's disease

To see whether eosinophils express GRO-α in vivo and in a context where these cells may have important roles, tumor tissue obtained from patients suffering from Hodgkin's disease of the nodular sclerosing type was investigated. The vast majority of the abundant GRO-α-containing cells showed distinct phenotypic features of those of eosinophils as detected by immunohistochemistry (Fig. 7*a*). Replacement of the specific primary Ab with an irrelevant isotype-matched Ab resulted in a loss of staining (Fig. 7*b*). In

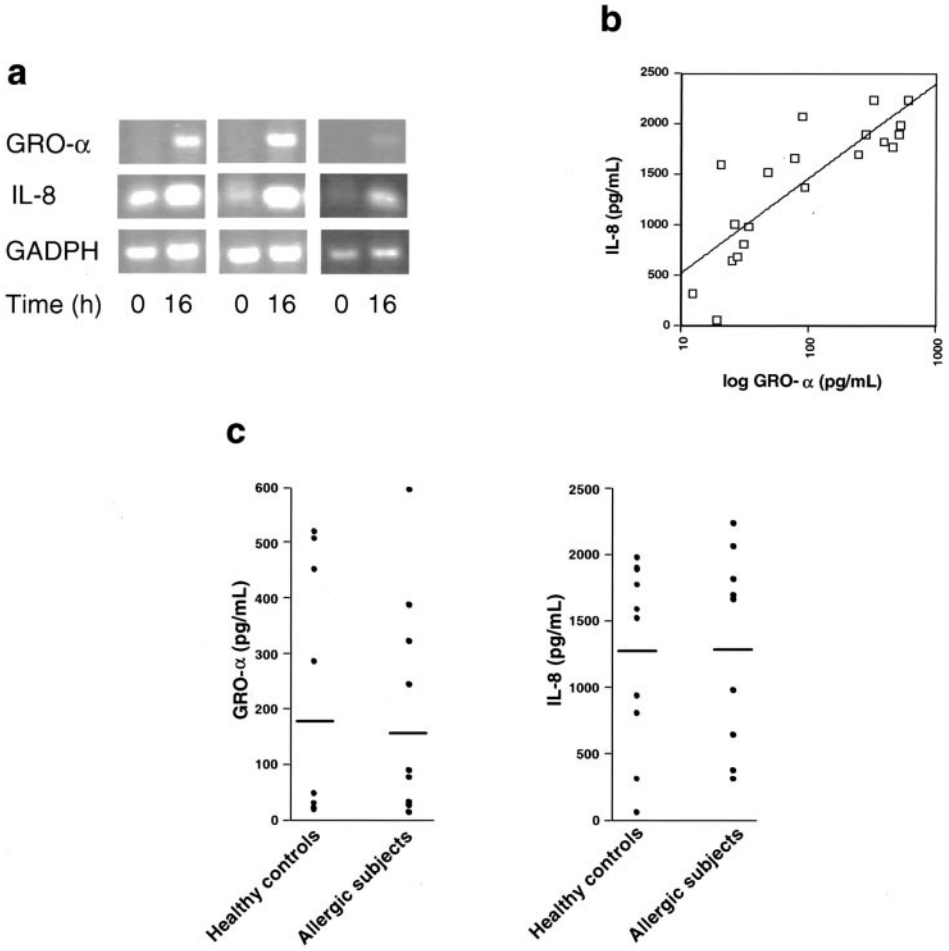

FIGURE 3. Correlation of GRO-α and IL-8 expression in eosinophils. *a*, Concomitant up-regulation of GRO-α and IL-8 in eosinophils during prolonged incubation. The level of GRO-α and of IL-8 expression was determined by RT-PCR in cells immediately after isolation and after 16 h of incubation. A low initial expression of GRO-α was sometimes detected whereas the expression of IL-8 was higher, although a large degree of variation was seen in between donors and was not related to the absence or presence of allergic disease. Three different donors are shown to demonstrate the variation in gene expression. *b*, Correlation between GRO-α and IL-8 release from eosinophils during prolonged incubation. Eosinophils were incubated in medium for 24 h. GRO-α and IL-8 were measured in the medium by ELISA. The data shown are from 20 separate experiments and donors. Linear regression analysis was used, and the correlation is highly significant ($p = 0.00038$). *c*, The expression of GRO-α and IL-8 in atopic and nonatopic subjects. Eosinophils were isolated from blood obtained from donors with confirmed seasonal rhinoconjunctivitis ($n = 10$) during pollen season and donors without a history of allergic disease or atopic constitution ($n = 10$). No differences were observed between the two groups with respect to the propensity to express GRO-α or IL-8 after 24 h of incubation.

situ hybridization showed an extensive presence of GRO-α-expressing cells (Fig. 7*c*). As a negative control for in situ hybridization, an irrelevant probe was used and resulted in the loss of labeling (data not shown). To confirm the eosinophil phenotype of the GRO-α expressing cells, the sections that had been subjected to in situ hybridization were also incubated with an Ab against the eosinophil granule protein ECP. Bound Ab was visualized by FITC-conjugated secondary Ab. There was a colocalization between cells containing ECP and those showing *GRO-α* gene expression (Fig. 7*d*).

The presence of the GRO-α receptor CXCR2 was demonstrated on the same tissue sections by immunohistochemistry (Fig. 7*e*). A large number of mononuclear cells showed presence of CXCR2, whereas eosinophils did not. Replacement of the specific primary Ab with an irrelevant isotype-matched Ab resulted in loss of staining (Fig. 7*f*).

Discussion

In the present study, we show that ELR$^+$ CXC chemokine GRO-α is expressed and released by human eosinophils. Preformed GRO-α was found in the specific granules, and increased production of GRO-α was observed during prolonged incubation. The strong increase in GRO-α expression in eosinophils was, at least in part, dependent on autocrine and paracrine stimulation by TNF-α produced by the cells. Furthermore, eosinophils present in tumor tissue from Hodgkin's disease showed expression of GRO-α, thus demonstrating occurrence in vivo during disease.

In addition to showing the cytotoxic properties of eosinophils, research in recent years has shown that eosinophils can produce several cytokines (2). Therefore, they are likely to have roles in orchestrating inflammation. Hitherto, IL-8 has been the only CXC chemokine known to be expressed in eosinophils (12, 15). Eosinophils also express four different CC chemokines, i.e., monocyte chemoattractant protein-1, MIP-1α, RANTES, and eotaxin (16–18). The levels of GRO-α and IL-8 reported in this study are relatively high compared with those reported for the CC chemokines (19, 20). Taken together, this suggests that eosinophils express CXC chemokines in favor of CC chemokines.

During inflammation, CXC chemokines seem to predominantly activate neutrophils. In addition, CXCRs are found on the surface of monocytes/macrophages, T cells, NK cells, mast cells, and endothelial cells but not on the surface of eosinophils (3, 21). The only characterized receptor for GRO-α is CXC chemokine receptor 2 (CXCR2), and activation of the receptor on neutrophils and mast cells result in migratory responses (22). Using gene-specific knockout mice, CXCR2 was shown to be essential for neutrophil recruitment to the cornea in helminth-mediated keratitis (river blindness) (23).

We found that eosinophils from different donors were heterogeneous with respect to their constitutive expression of GRO-α and IL-8. Increased expression of IL-8 in eosinophils has been shown in patients with allergy (12). In the present study, we could not relate the degree of constitutive expression to the absence or presence of allergic disease (data not shown). Nor were the amounts of GRO-α and IL-8 synthesized during prolonged incubation in vitro attributed to the absence or presence of ongoing allergic inflammation.

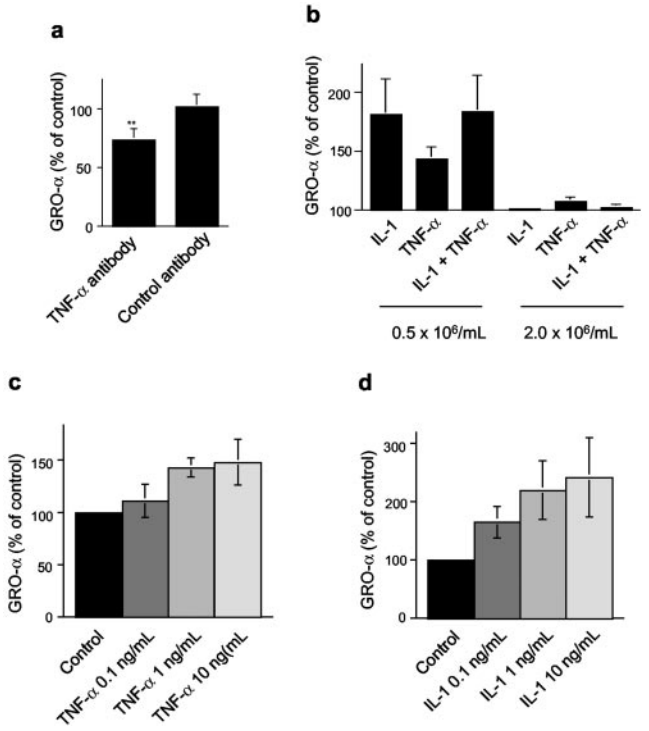

FIGURE 4. Effects from TNF-α and IL-1β on GRO-α expression in eosinophils. *a*, Autocrine and paracrine effects from TNF-α are important for the production of GRO-α during prolonged incubation. Eosinophils (2×10^6/ml) were incubated in medium alone, or in the presence of a TNF-α-neutralizing Ab (10 μg/ml) or in the presence of an isotype-matched control Ab (10 μg/ml) for 24 h. TNF-α-neutralizing Abs significantly ($p < 0.01$) inhibited the production of GRO-α. The data shown are expressed as percentage of GRO-α compared with the production by cells in medium alone. *b*, The autocrine and paracrine effects are saturated at a high but not at a lower cell density. Eosinophils were incubated at two different densities (0.5×10^6/ml and 2×10^6/ml, respectively) in medium alone, or in the presence of IL-1β (5 ng/ml), TNF-α (5 ng/ml), or both cytokines. At the lower cell density, both IL-1β and TNF-α increased the production of GRO-α measured by ELISA. No additive or synergistic effects were observed. At the higher cell density, there was a negligible increase of the GRO-α after addition of the cytokines. The data shown are normalized against the GRO-α produced by eosinophils incubated in medium alone at the respective cell densities and represent mean ± SEM from four separate experiments. *c*, The effect from TNF-α is dose dependent. Eosinophils (0.5×10^6/ml) were incubated for 24 h in medium containing increasing concentrations of TNF-α (0.1, 1.0, and 10 ng/ml). The amount of released GRO-α was measured using ELISA. The results are presented as percentage of control. The data shown represent means ± SEM from three separate experiments. *d*, A higher dose of IL-1β results in increased release of GRO-α from eosinophils, demonstrating a dose-dependent response. Cells were incubated at a low cell concentration (0.5×10^6/ml) in medium in the presence of IL-1β at different concentrations (0.1, 1.0, and 10 ng/ml) for 24 h. The amount of released GRO-α was measured by ELISA. The data shown are normalized against the GRO-α produced by eosinophils incubated in medium alone and represent means ± SEM from three separate experiments.

TNF-α is important for the up-regulation of *GRO-α* gene expression in eosinophils in vitro. Eosinophils can produce TNF-α and possess its corresponding receptors, making autocrine and paracrine stimulation possible (16, 24). At sites of inflammation, neighboring cells, such as macrophages, may promote *GRO-α* gene expression in eosinophils by producing TNF-α as well as IL-1. IL-1 has also been shown to induce the expression of GRO-α (13).

IFN-γ strongly inhibited the production of GRO-α in eosinophils, which is similar to the effect of IFN-γ on *GRO-α* gene expression in

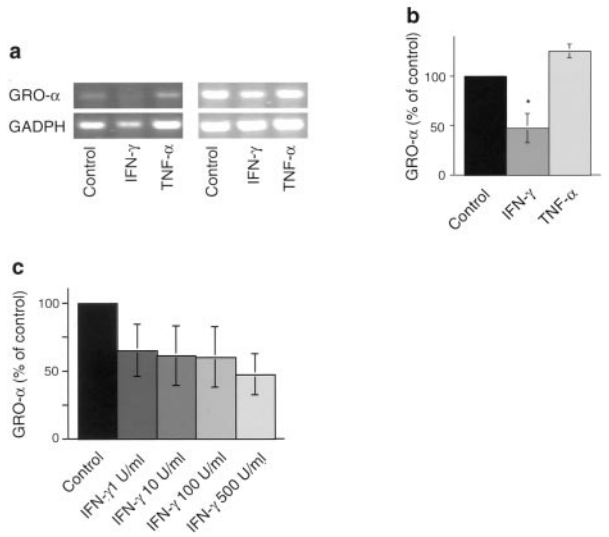

FIGURE 5. IFN-γ down-regulates GRO-α production in eosinophils. *a*, IFN-γ down-regulates the expression, whereas there is a tendency of up-regulation with TNF-α. RNA was isolated from eosinophils after 24 h of incubation in medium alone or in the presence of IFN-γ (500 U/ml) or TNF-α (5 ng/ml) and analyzed with RT-PCR. Two representative donors are shown. *b*, IFN-γ caused a decrease in GRO-α synthesis ($p < 0.01$), whereas TNF-α showed a nonsignificant effect toward a stimulatory effect. Eosinophils were incubated for 24 h in medium alone or in the presence of IFN-γ (500 U/ml) or TNF-α (5 ng/ml). The amounts of GRO-α were measured by ELISA. The data are presented as percentage of control (cells in medium alone) and represent means ± SEM from four separate donors and experiments. *c*, The inhibitory effect from IFN-γ was dose dependent. Eosinophils were incubated 24 h in medium containing increasing concentrations of IFN-γ. The data shown are normalized against the GRO-α produced by eosinophils incubated in medium alone and represent means ± SEM from four separate experiments.

monocytes and in neutrophils (25). Important roles for eosinophils during inflammation can be recruitment and activation of neutrophils. At early stages of inflammation, neutrophils dominate but are gradually replaced by monocytes and lymphocytes. Therefore, IFN-γ

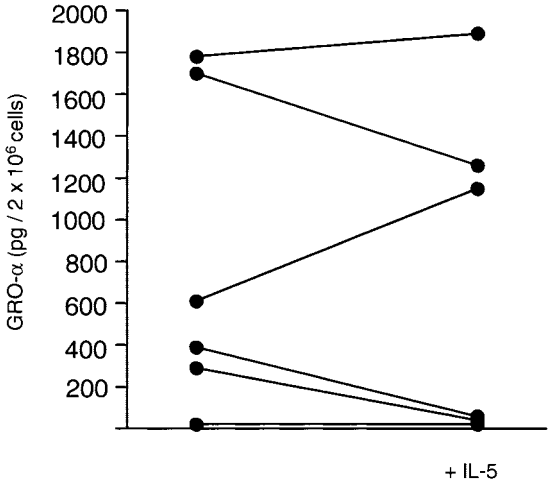

FIGURE 6. Effect from the eosinophil-activating cytokine IL-5. IL-5 showed both stimulatory and inhibitory effect on GRO-α production. Eosinophils were isolated from 33 separate donors, and incubated for 24 h in the absence or presence of IL-5 (1 nM). Released amounts of GRO-α were measured by ELISA. The figure shows six representative experiments.

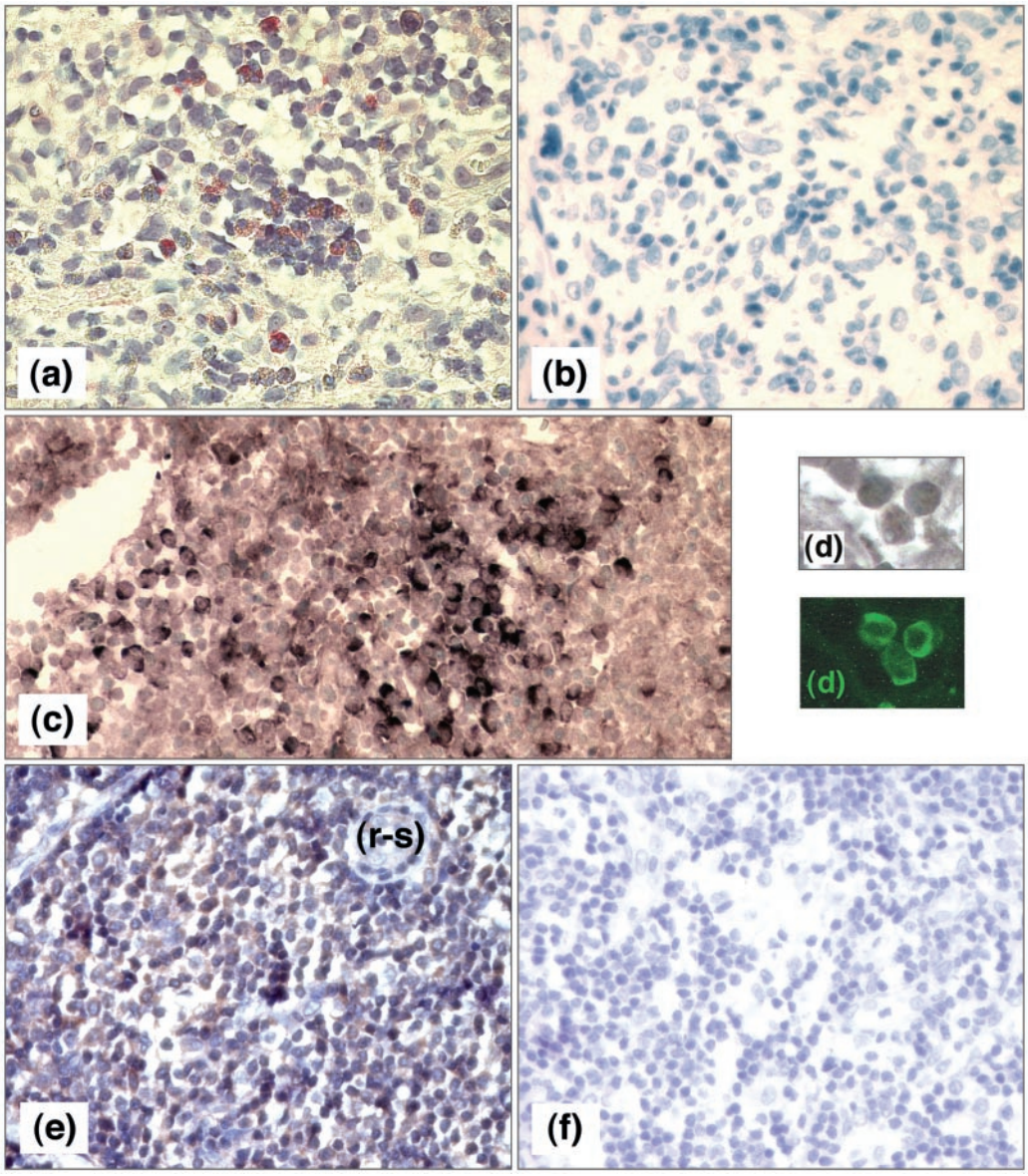

FIGURE 7. GRO-α is expressed by eosinophils present in tumor tissue from patients with Hodgkin's disease of the nodular sclerosing type. *a*, GRO-α detected by immunohistochemistry in tumor tissue from a patient with Hodgkin's disease. Abundant presence of cells containing GRO-α is seen. These cells show features typical of eosinophils, i.e., coarse granular cytoplasm and bilobed nuclei. Magnification, $\times$40. *b*, As a negative control, the GRO-α-specific Ab was replaced by an irrelevant isotype-matched Ab, which results in loss of immunoreactivity. Magnification, $\times$40. *c*, *GRO-α* gene expression on a single-cell level as detected by in situ hybridization. Presence of GRO-α mRNA is detected by hybridization with a specific oligonucleotide which is detected by an alkaline phosphatase reaction resulting in a dark-violet staining of the cytoplasm and an abundance of GRO-α expressing cells. *d*, The same section as shown in *c* was incubated with a specific Ab against the eosinophil granule protein ECP and detected by a secondary FITC-conjugated Ab (*lower panel*), confirming the phenotypic identity of eosinophils in the tumor tissue. Shown are the same cells as in *c*. Magnification, $\times$40. *e*, The presence of the GRO-α receptor CXCR2 in tumor tissue as detected by immunohistochemistry. An abundance of labeled cells is seen, but no staining of eosinophils is seen. r-s, Reed-Sternberg cell; magnification, $\times$40. *f*, The use of an irrelevant isotype-matched Ab resulted in a loss of labeling; magnification, $\times$40 (22).

produced by macrophages or T lymphocytes, may down-regulate the recruitment of inflammatory cells by suppressing the release of neutrophil-attracting chemokines, such as GRO-α (25). IL-5 is an eosinophil-activating cytokine, delaying eosinophil apoptosis and up-regulating several cellular functions (1). During the experimental conditions used, IL-5 showed varying effects on GRO-α production from eosinophils. Therefore, other factors may interact with IL-5, suggesting a complex regulation of GRO-α production during allergic inflammation.

GRO-α was initially described as melanoma growth stimulatory factor and isolated as a peptide characterized by its mitogenic effects (5). Ablation of eosinophils has been shown to retard tumor growth in an experimental model. In another model, both tumor growth and angiogenesis was dependent on CXCR2 activation (26, 27). Taken together, this suggests that CXC chemokine expression by eosinophils may play a role in tumor growth. In Hodgkin's disease of the nodular sclerosing type, it has been shown that a higher degree of eosinophilia in the tumor tissue strongly correlates with a poor prognosis (28). The tumor cells, i.e., the Reed-Sternberg cells of Hodgkin's disease, express the eosinophil-specific cytokine IL-5, which may explain the recruitment and activation of eosinophils (29, 30). We show abundant expression of GRO-α by eosinophils infiltrating tumor tissue in Hodgkin's disease. In addition, a large number of CXCR2-bearing cells were

present in the tumors. However, <1% of such tumors are composed of malignant cells, the remaining cells being B and T lymphocytes, eosinophils, macrophages, and dendritic cells, suggesting a complex network of interacting cells (31).

In conclusion, we show that eosinophils, through autocrine and paracrine stimulation by TNF-α, produce large amounts of the CXC chemokine GRO-α. This suggests proinflammatory roles for eosinophils during inflammation. Our finding may also be of importance in explaining an interaction between eosinophils and certain tumors.

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