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The CXC Chemokine GCP-2/CXCL6 Is Predominantly Induced in Mesenchymal Cells by Interleukin-1β and Is Down-Regulated by Interferon-γ: Comparison with Interleukin-8/CXCL8

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SUMMARY: Human granulocyte chemotactic protein-2 (GCP-2)/CXCL6 is a CXC chemokine that functionally uses both of the IL-8/CXCL8 receptors to chemoattract neutrophils but that is structurally most related to epithelial cell–derived neutrophil attractant-78 (ENA-78)/CXCL5. This study provides the first evidence that GCP-2 protein is, compared with IL-8, weakly produced by some sarcoma, but less by carcinoma cells, and is tightly regulated in normal mesenchymal cells. IL-1β was the predominant GCP-2 inducer in fibroblasts, chondrocytes, and endothelial cells, whereas IL-8 was equally well up-regulated in these cells by TNF-α, measles virus, or double-stranded RNA (dsRNA). In contrast, lipopolysaccharide (LPS) was a relatively better stimulus for GCP-2 versus IL-8 in fibroblasts. IFN-γ down-regulated the GCP-2 production in fibroblasts induced by IL-1β, TNF-α, LPS, or dsRNA. The kinetics of GCP-2 induction by IL-1β, LPS, or dsRNA in fibroblasts differed from those of IL-8. Freshly isolated peripheral blood mononuclear leukocytes, which are a good source of IL-8 and ENA-78, failed to produce GCP-2. However, lung macrophages and blood monocyte–derived macrophages produced GCP-2 in response to LPS. Quantitatively, secretion of GCP-2 always remained inferior to that of IL-8, despite the fact that the ELISA recognized all posttranslationally modified GCP-2 isoforms. The expression of GCP-2 was confirmed in vivo by immunohistochemistry. The patterns of producer cell types, inducers and kinetics and the quantities of GCP-2 produced, suggest a unique role for GCP-2 in physiologic and pathologic processes. (*Lab Invest 2003, 83:23–34*).

Chemokines constitute a large family of chemotactic cytokines that regulate cell migration during physiologic and inflammatory processes. They are important for host defense, but they also have fundamental roles in the development and homeostasis of the immune system. In addition, chemokines are involved in angiogenesis and tumorigenesis (Baggiolini, 1998; Luster, 1998; Mantovani, 1999; Zlotnik and Yoshie, 2000). Based on the position of conserved cysteines, chemokines are divided into four subfamilies: CXC, CC, C, and CX_3C chemokines (Baggiolini, 1998; Luster, 1998; Mantovani, 1999; Zlotnik et al, 1999; Zlotnik and Yoshie, 2000). The CXC chemokines, containing one amino acid between the two NH_2-terminal cysteine residues, can further be divided into two subgroups depending on the presence or absence of the sequence Glu-Leu-Arg (ELR) immediately in front of the first cysteine residue. The ELR^+CXC chemokines GROα/CXCL1, GROβ/CXCL2, GROγ/CXCL3, epithelial cell–derived neutrophil attractant-78 (ENA-78)/CXCL5, granulocyte chemotactic protein-2 (GCP-2)/CXCL6, neutrophil-activating protein-2 (NAP-2)/CXCL7, and IL-8/CXCL8 chemoattract and activate neutrophils, whereas the ELR^-CXC chemokines are mainly active on lymphocytes (Luster, 1998; Zlotnik et al, 1999; Zlotnik and Yoshie, 2000). In addition, ELR^+CXC chemokines induce endothelial cell chemotaxis in vitro and are angiogenic in vivo (Strieter et al, 1995).

Chemokines can be secreted by most cell types. Many chemokines are only produced after appropriate stimulation of the cells with proinflammatory cytokines or bacterial or viral products (inducible chemokines). However, some chemokines are constitutively produced (constitutive chemokines) (Mantovani, 1999;

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Zlotnik and Yoshie, 2000). Whereas the constitutive chemokines mainly have homeostatic functions, the inducible chemokines are important during inflammatory reactions.

Human IL-8 and GCP-2 are the only ELR$^+$CXC chemokines that recognize both the receptors CXCR1 and CXCR2 (Murphy et al, 2000; Wolf et al, 1998; Wuyts et al, 1998a). IL-8 is the first identified member of this chemokine subgroup and is the most potent neutrophil chemotactic and activating protein (Wuyts et al, 1998b). IL-8 production in response to a variety of inducers has been observed for almost all cell types (Wuyts et al, 1998b). GCP-2 was originally isolated from conditioned medium of cytokine-stimulated osteosarcoma cells (Proost et al, 1993) and shows the highest structural homology with the ELR$^+$CXC chemokine ENA-78 (77% identical amino acids). The latter was discovered as a neutrophil-attracting chemokine produced by pulmonary epithelial cells stimulated with IL-1β or TNF-α (Walz et al, 1991). The gene regulation of ENA-78 has already been investigated in detail (Walz, 2000; Walz et al, 1997). Preliminary data on mRNA expression of GCP-2 in some cell types were obtained by semiquantitative RT-PCR (Froyen et al, 1997), but regulated production of GCP-2 protein in different normal cell types has not been studied yet.

Similar to IL-8 and ENA-78, GCP-2 occurs in different molecular forms as a result of NH_2-terminal truncation. To study the gene regulation of GCP-2, a specific ELISA, which recognizes the different natural forms, was developed. We found that, both quantitatively and qualitatively, the production of IL-8 and GCP-2 differed. For GCP-2, the amounts of protein induced were lower and the spectrum of producer cell types was more limited than for IL-8. GCP-2 was predominantly induced in mesenchymal cells by IL-1β but down-regulated by IFN-γ. This suggests a more selective role of GCP-2 in inflammation. Furthermore, the expression of GCP-2 was confirmed in vivo by immunohistochemistry on intestinal biopsy samples.

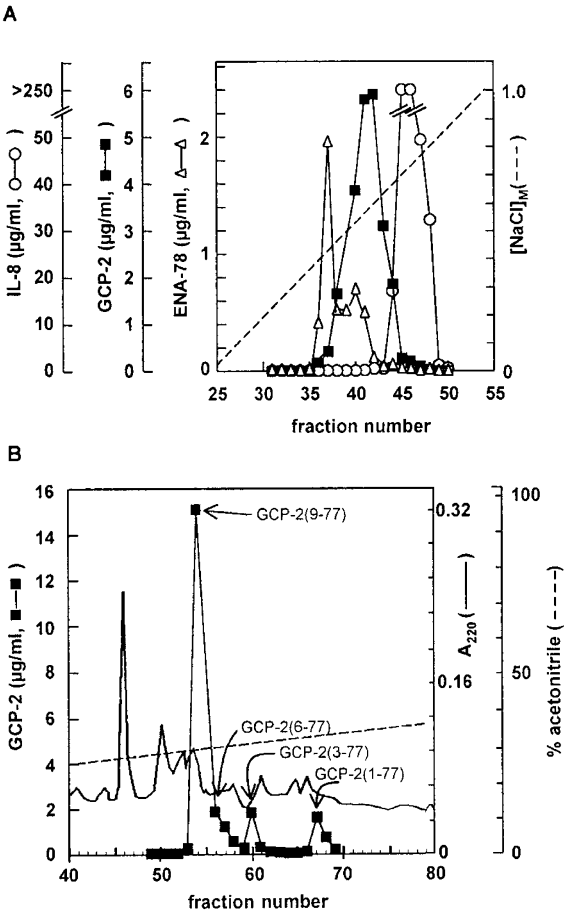

Figure 1.

Identification of immunoreactive GCP-2 isoforms from human osteosarcoma cell conditioned medium. A, Natural human GCP-2, concentrated and partially purified from MG-63 osteosarcoma cell conditioned medium, was subjected to cation-exchange chromatography. Proteins were eluted with a linear NaCl gradient, and the fractions were analyzed by specific ELISAs for the presence of IL-8, GCP-2, or ENA-78 immunoreactivity. B, GCP-2 immunoreactivity, eluted from the cation-exchange column, was further purified by RP-HPLC. Proteins were eluted with an acetonitrile gradient and detected by their UV absorbance at 220 nm. Subsequently, RP-HPLC fractions were subjected to the GCP-2 ELISA. The identity of the GCP-2 isoforms was determined by NH_2-terminal amino acid sequence analysis.

Results

Nondiscriminative Detection of Multiple Natural Isoforms of Human GCP-2 by a Specific ELISA

A specific ELISA for GCP-2 was developed, using a polyclonal anti-human GCP-2 antibody for coating and a monoclonal anti-human GCP-2 as capturing antibody. To evaluate the specificity of the GCP-2 ELISA, natural human GCP-2 was purified in parallel with other chemokines from MG-63 osteosarcoma cell conditioned medium by a four-step purification procedure (adsorption to controlled pore glass beads, antibody affinity chromatography, cation-exchange fast protein liquid chromatography [FPLC] and reversed phase [RP]-HPLC). Fractions eluting from the cation-exchange column were subjected to the GCP-2 ELISA and to an IL-8 and ENA-78 ELISA. Figure 1A shows that GCP-2 elutes from the FPLC column as a single peak at 0.6 M NaCl, in front of IL-8 (eluting at 0.75 M NaCl) and after ENA-78 (eluting at 0.45 M) and GRO (data not shown). GCP-2 was further purified to homogeneity by RP-HPLC. Analysis of the obtained HPLC fractions in the GCP-2 ELISA yielded three peaks of GCP-2 immunoreactivity, corresponding to intact GCP-2(1-77) and different NH_2 terminally truncated forms—GCP-2(9-77), GCP-2(6-77), and GCP-2(3-77)—as identified by amino acid sequencing (Fig. 1B). In addition to intact GCP-2(1-77), the naturally processed isoforms of GCP-2 missing 2, 5, or 8 NH_2-terminal residues were also recognized by the ELISA. Furthermore, the amount of detected immunoreactivity corresponded to the concentration of the different GCP-2 forms deduced from protein detection by SDS-PAGE. In addition, the FPLC fractions containing ENA-78 and GRO were further purified by RP-HPLC. No GCP-2 immunoreactivity was detected in HPLC fractions containing natural GROα(1-73), GROγ(1-73), ENA-78(7-78), or ENA-78(1-78), as de-

termined by NH_2-terminal amino acid sequence analysis. Furthermore, conditioned medium of monocytes, freshly isolated from peripheral blood and stimulated for chemokine production with conA and lipopolysaccharide (LPS), was purified by a similar four-step purification procedure (adsorption to controlled pore glass beads, heparin-Sepharose affinity chromatography, cation-exchange FPLC, and RP-HPLC). This yielded homogeneous NAP-2, GROα(4,5,6-73), GROγ(5-73) and ENA-78(8,9-78). None of these natural chemokine forms was detected in the GCP-2 ELISA. The inducers used to stimulate chemokine production did not crossreact in the ELISA. In addition, CXCL8, CXCL5, CXCL10, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL17, and CCL20 at more than 250 ng/ml were not recognized by the GCP-2 ELISA, whereas 8 pg/ml of GCP-2 was still detectable. These data indicate both the specificity and the sensitivity of this immunotest for GCP-2. These latter two characteristics are crucial, when we take into account the low amounts of GCP-2 produced together with the high levels of other inflammatory chemokines such as IL-8 (vide infra).

Production of GCP-2 by Tumor Cells

In view of its angiogenic activity and the fact that GCP-2 was originally purified from tumor cell–conditioned medium, the regulation of GCP-2 production by invasive tumor cell lines was first evaluated. MG-63 osteosarcoma cells showed a weak increase in GCP-2 production after stimulation with IL-1β, whereas PMA, measles virus, the double-stranded RNA (dsRNA) poly(riboinosinic acid).poly(ribocytidylic acid) (poly rI:rC), IFN-γ, and TNF-α had no effect (Fig. 2A). Similar to GCP-2, IL-8 was induced in these cells by IL-1β but not by poly rI:rC or IFN-γ. In contrast to GCP-2, IL-8 was inducible by PMA, measles virus, and TNF-α (Fig. 2A). The amount of IL-8 induced in MG-63 cells was about 1000-fold higher than that of GCP-2. Similar to MG-63 osteosarcoma cells, Malavu hepatosarcoma cells (not shown), HeLa cervix carcinoma cells (Fig. 2B), and IGROV ovarian carcinoma cells (not shown) produced IL-8 in response to PMA, measles virus, and IL-1β, whereas IFN-γ was not effective. PMA and to a lesser extent poly rI:rC induced IL-8 in Bowes melanoma cells (Fig. 2C). The amount of IL-8 produced by HeLa, Bowes, and Malavu cells was comparable to that secreted by MG-63 cells. However, none of the inducers tested were able to induce GCP-2 in these tumor cells (Fig. 2, B and C and not shown).

Production of GCP-2 by Normal Mesenchymal Cells

Because the angiogenic chemokine GCP-2 did not seem to be a major chemokine produced by tumor cells themselves, its production by normal cells surrounding tumor tissue was evaluated. Diploid fibroblasts, endothelial cells, and chondrocytes isolated from human embryonic skin and muscle, umbilical vein, and articular cartilage, respectively, were grown to confluency in vitro and were stimulated with various chemokine inducers for GCP-2 production. In contrast to tumor cells, fibroblasts showed a significant GCP-2 production (up to 10 ng/ml) after stimulation with PMA, measles virus, poly rI:rC, IL-1β, and TNF-α but not with IFN-γ (Fig. 3A). The production pattern in fibroblasts resembled that of IL-8. However, IL-1β was clearly the best inducer for GCP-2, whereas for IL-8 induction, IL-1β, TNF-α, measles virus, and poly rI:rC were equally active. This is in agreement with the finding that in MG-63 osteosarcoma cells the highest GCP-2 levels were induced by IL-1β (Fig. 2A). However, compared with the GCP-2 concentrations reached in MG-63 cells, those from diploid fibroblasts were 20-fold higher, whereas the IL-8 levels were similar for both cell types.

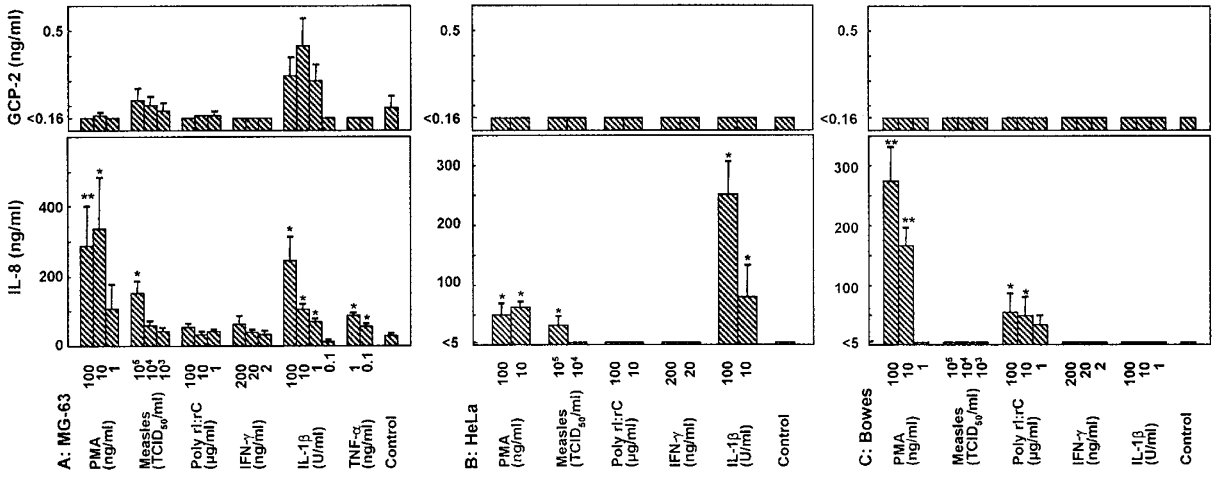

Figure 2.

Induction of GCP-2 and IL-8 in different tumor cell types. Human MG-63 osteosarcoma cells (A), HeLa cervix carcinoma cells (B), and Bowes melanoma cells (C) were grown to confluency and stimulated for 48 hours with several chemokine inducers at different concentrations or were left untreated (*Control*). IL-8 and GCP-2 immunoreactivity present in the supernatants were measured by specific ELISAs. Results represent the mean ± SEM of two to five independent experiments. Asterisks indicate a significant chemokine induction compared with the untreated cells. $*p < 0.05$; $**p < 0.01$.

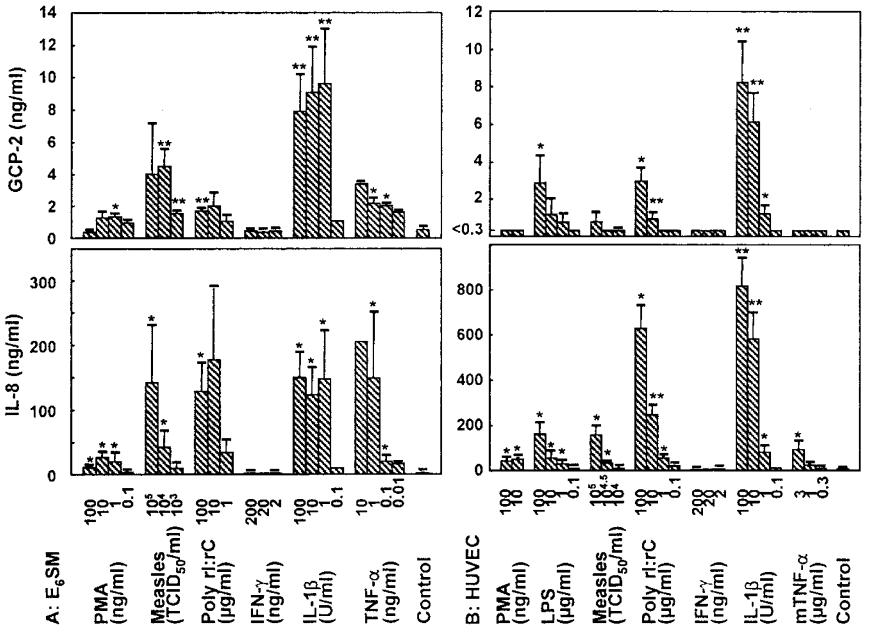

Figure 3.

Production of GCP-2 and IL-8 by normal mesenchymal cells. Human diploid skin-muscle fibroblasts (*E_6SM*) (A) and human umbilical vein endothelial cells (HUVEC) (B) were grown to confluency and stimulated for 48 hours with several inducers at different concentrations or were left untreated (*Control*). IL-8 and GCP-2 immunoreactivity present in the supernatants were measured by specific ELISAs. Results represent the mean ± SEM of two to five independent experiments. Asterisks indicate a significant chemokine induction compared with the untreated cells. $*p < 0.05$; $**p < 0.01$.

As shown for fibroblasts, IL-1β and poly rI:rC dose-dependently induced GCP-2 protein in endothelial cells (Fig. 3B). In addition, GCP-2 production was stimulated by LPS, whereas PMA and TNF-α were ineffective. IL-8 protein was clearly coinduced by IL-1β, poly rI:rC, and LPS, but was also weakly stimulated by measles virus, TNF-α, and PMA. Again, IL-1β was the best inducer for GCP-2, whereas for IL-8, poly rI:rC and IL-1β were equally inducive. No GCP-2 or IL-8 was measured after stimulation of the cells with IFN-γ. The amount of GCP-2 inducible in endothelial cells was comparable to that produced by fibroblasts. The levels of induced IL-8 were about 100 times higher than those for GCP-2.

Finally, chondrocytes were found to secrete both IL-8 and GCP-2 in response to IL-1β, TNF-α, LPS, and poly rI:rC. However, the amounts of IL-8 produced were again about 100-fold higher than those of GCP-2 (Fig. 4). Taken together, IL-1β was the most effective inducer of GCP-2 in various connective tissue cells.

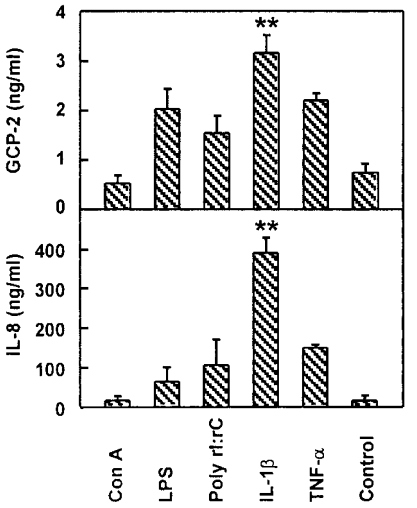

Figure 4.

Production of GCP-2 and IL-8 by chondrocytes. Human chondrocytes were stimulated for 24 hours with several inducers (10 μg/ml conA, 10 μg/ml lipopolysaccharide [*LPS*], 10 μg/ml poly rI:rC, 5 U/ml IL-1β, or 10 ng/ml TNF-α) or were left untreated (*Control*). IL-8 and GCP-2 immunoreactivity present in the supernatants were measured by specific ELISAs. Results represent the mean ± SEM of two to five independent experiments. Asterisks indicate a significant chemokine induction compared with the untreated cells. $**p < 0.01$.

Production of GCP-2 by Hematopoietic Cells

Granulocytes, the target cells of GCP-2, were shown to produce various cytokines and CXC chemokines, eg, IL-8 (Cassatella, 1999). We could not detect GCP-2 in supernatants of these cells, although we confirmed production of IL-8 by granulocytes (about 150 ng/ml, not shown) in response to LPS.

Resident or recruited macrophages often contribute to the inflammatory response in normal or tumoral tissues by the secretion of chemokines. After induction with LPS, pulmonary macrophages freshly isolated from resections of patients undergoing surgery for lung carcinoma produced IL-8 and GCP-2 (Fig. 5A). Similar results were obtained when monocytes isolated from peripheral blood were enriched by adherence to plastic (>90% monocytes). When allowed to differentiate into macrophages by in vitro culture for 5 days, LPS but not poly rI:rC or IL-1β induced GCP-2 (0.4 ng/ml) and IL-8 (400 ng/ml) (Fig. 5B). In contrast,

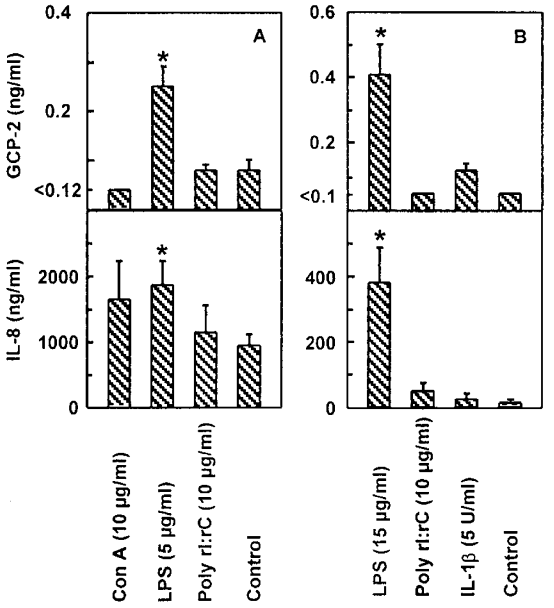

Figure 5.

Production of GCP-2 by macrophages. Freshly isolated pulmonary macrophages (A) or macrophages differentiated for 5 days in vitro from adherent blood monocytes (B) were stimulated for 72 hours with several inducers or were left untreated (*Control*). IL-8 and GCP-2 immunoreactivity present in the supernatants were measured by specific ELISAs. Results represent the mean ± SEM of three to five independent experiments. Asterisks indicate a significant chemokine induction compared with the untreated cells. $*p < 0.05$.

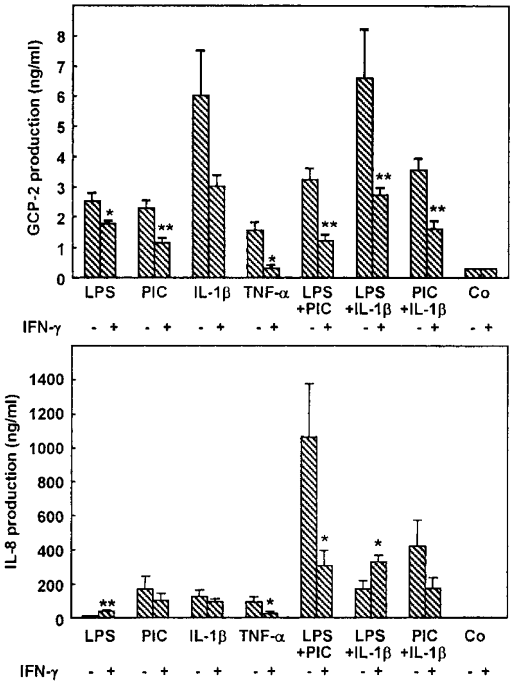

Figure 6.

Effects of IFN-γ on GCP-2 and IL-8 induction in fibroblasts. Fibroblasts (E_6SM or E_1SM) were grown to confluency and stimulated for 68 hours with LPS (50 µg/ml), poly rI:rC (*PIC*; 100 µg/ml), IL-1β (10 U/ml), TNF-α (10 ng/ml), or a combination of those inducers in the absence or presence of IFN-γ (200 ng/ml). IL-8 and GCP-2 immunoreactivity present in the supernatants were measured by specific ELISAs. Results represent the mean ± SEM of five independent experiments. Asterisks indicate a significant decrease or increase of chemokine production in the presence of IFN-γ compared with production in the absence of IFN-γ. $*p < 0.05$; $**p < 0.01$.

mononuclear leukocytes freshly isolated from peripheral blood (<20% monocytes) and immediately stimulated without a prior maturation period failed to produce GCP-2, whereas IL-8 was produced in significant amounts (up to 500 ng/ml) in response to conA, LPS, and IL-1β (vide infra: kinetics of GCP-2 production). This indicates that both the inducer and the differentiation stage of the stimulated cells influence GCP-2 production and suggests that the presence of this chemokine can depend on the type and phase of disease.

Down-Regulation of GCP-2 Production in Fibroblasts by IFN-γ

Because IL-1β has been reported to synergize with other cytokines such as IFN-γ (Menten et al, 1999; Struyf et al, 1998), which failed to induce GCP-2, the effect of simultaneous addition of different stimuli on GCP-2 production by fibroblasts was evaluated. First, these experiments confirm the induction of GCP-2 and IL-8 protein in fibroblasts (Figs. 3 and 6). Furthermore, LPS, which is not a standard inducer of chemokines in fibroblasts, also stimulated GCP-2 protein production as efficiently as poly rI:rC and TNF-α, whereas it was only an inferior inducer of IL-8 (Fig. 6). Addition of IFN-γ to the individual or combined inducers diminished the GCP-2 production in response to poly rI:rC, LPS, IL-1β, and TNF-α. This decrease in GCP-2 production by IFN-γ ranged from approximately 30% to 80%. For comparison, the TNF-α–induced IL-8 production by fibroblasts was diminished by IFN-γ, whereas the poly rI:rC-induced and IL-1β–induced production were not significantly affected (Fig. 6). In contrast to the inhibitory effect of IFN-γ on LPS-induced GCP-2 production, the LPS-induced IL-8 production was enhanced 3-fold by IFN-γ. No synergistic effect was observed for GCP-2 production in response to any combination of LPS, poly rI:rC, or IL-1β, whereas LPS and poly rI:rC synergistically induced IL-8 ($p < 0.01$). This IL-8 production was diminished by IFN-γ, whereas IFN-γ enhanced the IL-8 production stimulated by a combination of LPS and IL-1β (Fig. 6). These data clearly demonstrate that IL-8 and GCP-2 are differently regulated upon combined treatment with bacterial and viral products and that IFN-γ has distinct modulating effects on IL-8 and GCP-2 production by stimulated fibroblasts.

Kinetics of GCP-2 Production

Because only low amounts of GCP-2 were produced by cells stimulated for 48 hours, more detailed experiments were performed to study the kinetics of GCP-2 production. Diploid fibroblasts, MG-63 osteosarcoma cells, and mononuclear leukocytes were stimulated with LPS, conA, poly rI:rC, or IL-1β. These experiments confirmed that in MG-63 osteosarcoma cells, GCP-2 is induced only by IL-1β but not by LPS or poly rI:rC (Fig. 7A). Although IL-1β already stimulated sig-

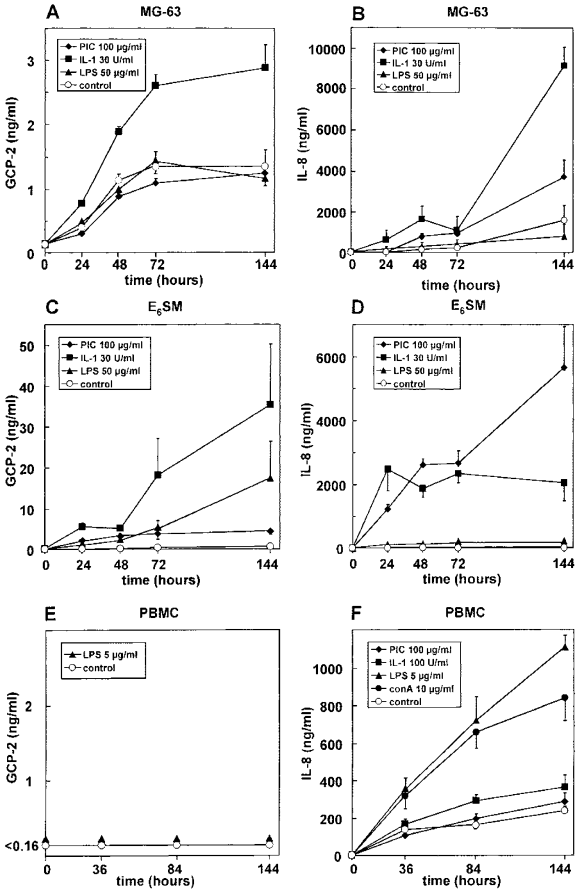

Figure 7.

Kinetics of GCP-2 and IL-8 production in osteosarcoma cells, fibroblasts, and mononuclear leukocytes. MG-63 osteosarcoma cells (A and B) and E_6SM fibroblasts (C and D) were grown to confluency in 25-cm^2 flasks and stimulated with LPS, poly rI:rC (*PIC*), or IL-1β. Peripheral blood mononuclear cells (E and F) were isolated from peripheral blood, seeded in 25-cm^2 flasks (5×10^6 cells/ml) and stimulated with conA, LPS, poly rI:rC (PIC), or IL-1β. Samples were taken at different time points and analyzed for the presence of GCP-2 (A, C, and E) and IL-8 (B, D, and F) immunoreactivity by specific ELISAs. Results represent the mean $\pm$ SEM of three independent experiments.

nificant GCP-2 expression after 24 hours, the production continued to reach a plateau after 72 hours. Even after 6 days, poly rI:rC and LPS remained ineffective to induce GCP-2. In contrast, IL-8 production by poly rI:rC and IL-1β increased until 144 hours after induction (Fig. 7B).

In fibroblasts, GCP-2 was already significantly induced after 24 hours of stimulation with IL-1β (Fig. 7C). The IL-1β-induced production continued until 144 hours after induction, whereas poly rI:rC-induced GCP-2 levels remained weak and reached a plateau after only 48 hours. LPS induced GCP-2 protein production with similar kinetics as IL-1β, indicating a direct stimulating effect of LPS on GCP-2 transcription. IL-8 production by fibroblasts in response to IL-1β and poly rI:rC was already high after 24 hours of induction (Fig. 7D). In contrast to GCP-2, the IL-8 concentration in fibroblasts after stimulation with IL-1β did not increase further after 24 hours, whereas the poly rI:rC-induced production continued. LPS was a marginal inducer of IL-8 compared with IL-1β and poly rI:rC (Fig. 7D).

In conditioned medium of stimulated mononuclear cells freshly isolated from peripheral blood, no GCP-2 production was detected after stimulation with conA (10 μg/ml), LPS (5 or 15 μg/ml), poly rI:rC (100 μg/ml), or IL-1β (100 U/ml), even after 144 hours (Fig. 7E and not shown). In contrast, IL-8 production in these cells in response to conA and LPS continued until Day 6 (Fig. 7F). Compared with conA and LPS, IL-1β was a rather weak inducer of IL-8 during the whole period of time and poly rI:rC was ineffective.

It can be concluded that GCP-2 induction by IL-1β was prolonged in fibroblasts for 6 days, whereas IL-1β–induced IL-8 levels were already maximal after 1 day, suggesting a more specific role of GCP-2 in prolonged inflammation. In addition, the delayed induction of GCP-2 in fibroblasts by LPS versus the continued induction of IL-8 by dsRNA points to a differential role of these two neutrophil chemoattractants in bacterial versus viral infection. Furthermore, the strong induction of IL-8, and not GCP-2, by LPS in fresh mononuclear leukocytes and the high production of GCP-2, and not IL-8, by LPS-induced fibroblasts indicate a complementary production of these two CXC chemokines. Finally, the kinetics of GCP-2 induction was different in normal and tumor cells.

Expression of GCP-2 Protein in the Intestine

Because GCP-2 protein is produced in vitro by mesenchymal cells and macrophages after stimulation with LPS or IL-1β, the presence of GCP-2 protein was evaluated in intestinal tissue to confirm expression in vivo (Fig. 8). Microscopic analysis of immunohistochemically stained cryostat sections showed the presence of small numbers of cells staining positive for GCP-2 in the mucosa (and occasionally in the submucosa) of biopsy specimens from patients with Crohn's disease (Fig. 8, A to C) and colon carcinoma (Fig. 8D). The positive staining appeared mainly as a finely granular or diffuse cytoplasmic staining. In the tissue sections obtained from patients with Crohn's disease, the number of GCP-2^+ cells was on average increased (5.9 ± 2.2 cells per 10 high-power magnification fields; $n = 10$) compared with the sections of normal colon from carcinoma patients (4.3 ± 1.7; $n = 4$). Cells showing a positive staining were essentially mononuclear cells in the lamina propria and were mainly present as single cells. Figure 8A shows the presence of an intestinal GCP-2^+ macrophage in a biopsy specimen from a patient with Crohn's disease after double immunostaining with the monocyte/macrophage marker HLA-DR. For this patient (Fig. 8A), the number of HLA-DR^+ GCP-2^+ cells was comparable with the number of HLA-DR^+ but GCP-2^- cells. Furthermore, the number of GCP-2-expressing cells was higher than the number of GCP-2^+ HLA-DR^+ cells, indicating that probably HLA-DR^- cells also can express GCP-2.

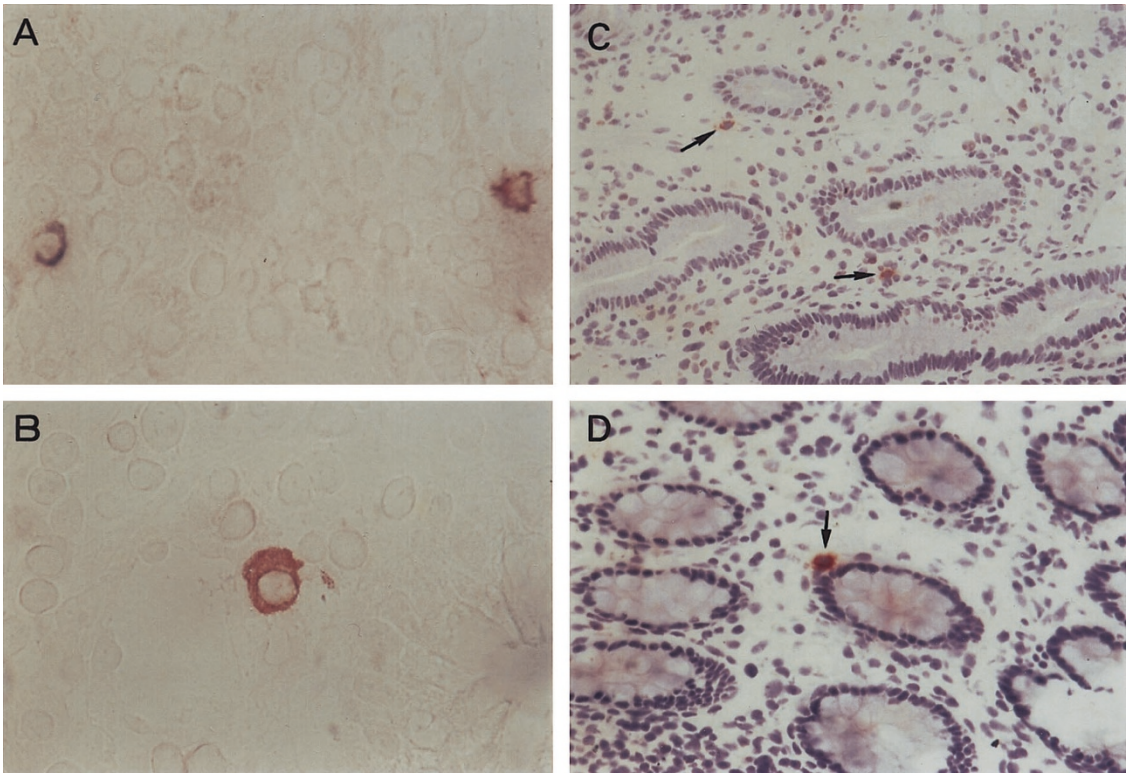

Figure 8.
A, Detection of GCP-2 protein in tissue biopsy specimens of the intestine. Intestinal tissue of a patient with Crohn's disease was analyzed by double immunohistochemical staining (without counterstaining) for GCP-2 and HLA-DR with 3-amino-9-ethylcarbazole (AEC) (bright red) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (black), respectively (magnification ×100). The black cell on the left indicates a monocyte/macrophage positive for HLA-DR, whereas the cell at the right stained positive for both HLA-DR and GCP-2 (reddish black color), indicating a GCP-2–producing monocyte/macrophage. B, A cell (without counterstaining) from a patient with Crohn's disease with positive cytoplasmic staining for GCP-2 with AEC (red) (×100). C, Illustrates immunohistochemical staining for GCP-2 in the small intestinal mucosa of a patient with Crohn's disease (×25). The *arrows* indicate GCP-2$^+$ cells in the lamina propria, whereas counterstaining reveals the morphology of the mucosa. D, The regular structure of crypts in normal tissue of a patient with colon carcinoma and a single GCP-2$^+$ cell (*arrow*) (×40).

Discussion

The expression of chemokines in various tissues is differently regulated during the immune response to infection. The production of the ELR$^+$CXC chemokines IL-8 (Wuyts et al, 1998b), GROα, β, and γ (Wang and Richmond, 2000), NAP-2 (Brandt et al, 2000), and ENA-78 (Walz, 2000) has already been studied in detail, but the gene regulation of GCP-2, especially at the protein level, had not been investigated yet. In this study we show that many tumor cells, ie, sarcoma, carcinoma, and melanoma cells, are poor producers of GCP-2. Only small amounts of GCP-2 (up to 3 ng/ml) were constitutively secreted or induced by IL-1β in MG-63 osteosarcoma cells, although natural GCP-2 was originally isolated from conditioned medium of cytokine-stimulated MG-63 cells (Proost et al, 1993). This can be explained by the fact that large volumes (liters) of conditioned medium were used as start material for purification of minute quantities of different natural GCP-2 isoforms. In addition to GCP-2, IL-8, and GRO, ENA-78, which is most homologous to GCP-2 (77% identical amino acids), was isolated in small amounts from the supernatant of these cytokine-stimulated osteosarcoma cells (Proost et al, 1993; Wuyts et al, 1999). The availability of various natural NH_2 terminally truncated forms of GCP-2 allowed confirmation that all of these were equally well recognized by our specific and sensitive GCP-2 ELISA.

It has previously been shown that IL-8 and ENA-78 are induced in mononuclear cells and neutrophils after stimulation with LPS (Baggiolini et al, 1994; Goodman et al, 1998; Rampart et al, 1992; Schnyder-Candrian and Walz, 1997; Strieter et al, 1992; Wuyts et al, 1998b). However, here we show that GCP-2 was not produced by freshly isolated mononuclear leukocytes nor by granulocytes in response to different inflammatory stimuli, including LPS. In contrast, pulmonary macrophages and in vitro–differentiated peripheral blood monocyte–derived macrophages produced detectable (0.4 ng/ml) amounts of GCP-2 upon stimulation with LPS. In addition, stimulated fibroblasts and endothelial cells produced up to 40 ng/ml of GCP-2. As previously shown for ENA-78 and IL-8 (Baggiolini et al, 1994; Strieter et al, 1992; Wuyts et al, 1998b), GCP-2 was found to be induced in fibroblasts by IL-1β. Although less pronounced, GCP-2 was also produced by these cells, in parallel with IL-8, in response to TNF-α, PMA, LPS, measles virus, and the dsRNA poly rI:rC. Like human GCP-2, murine GCP-2/LIX is also expressed in fibroblasts after stimulation with LPS (Smith and Herschman, 1995). Endothelial

cells produced GCP-2 and IL-8 after stimulation with IL-1β, dsRNA, or LPS. Similarly, ENA-78 production by endothelial cells has been shown after stimulation with IL-1β and LPS (Imaizumi et al, 1997; Lukacs et al, 1995; Strieter et al, 1992). These induction experiments suggest that GCP-2 is a typical mesenchymal cell–derived chemokine. In all cases, IL-1β was clearly the best inducer for GCP-2 production, whereas other inducers were equally effective for IL-8.

In contrast to the synergistic effect of IFN-γ on IL-1β–induced MCP-2 and MCP-3 production (Menten et al, 1999; Struyf et al, 1998), IFN-γ diminished the GCP-2 production in fibroblasts stimulated with IL-1β, TNF-α, dsRNA, and LPS. Similar to the GCP-2 induction, the IL-8 production induced by TNF-α was decreased by IFN-γ. These findings confirm the previously observed inhibition of TNF-α–induced IL-8 mRNA expression by IFN-β and IFN-γ in fibroblasts (Lee et al, 1990; Oliveira et al, 1992). Furthermore, IFN-γ has been shown to inhibit TNF-α–induced IL-8 production in endothelial cells (Nyhlén et al, 2000). In our study, no effect of IFN-γ was observed on IL-1β–stimulated IL-8 production in fibroblasts, whereas IFN-γ has been shown to inhibit IL-1–induced IL-8 production in epithelial cells (Galy and Spits, 1991), endothelial cells (Nyhlén et al, 2000), and monocytes (Schnyder-Candrian et al, 1995). IFN-γ attenuated the LPS-induced GCP-2 production, but it enhanced the LPS-induced IL-8 production in fibroblasts. In contrast, the production of IL-8 in response to LPS by monocytes was decreased by IFN-γ (Schnyder-Candrian et al, 1995). Furthermore, the synergistic production of IL-8 by LPS and dsRNA, which was not observed for GCP-2, indicates that very high concentrations of IL-8 may be present in connective tissue during coinfection with viruses and bacteria. These data clearly show a different regulation of IL-8 and GCP-2 production in fibroblasts. Finally, it cannot be excluded that other endogenous mediators constantly down-regulate the constitutive or inducible GCP-2 production, eg, IFN-β secreted by MG-63 osteosarcoma cells or by diploid fibroblasts. In that case, tumors deficient in secreting such inhibitory mediators could produce larger quantities of this chemokine.

Under most conditions (cell types, inducers) evaluated in this study, IL-8 was coproduced with GCP-2. Furthermore, the secreted IL-8 levels were in most cases at least 10 times higher compared with those of GCP-2. IL-8 and GCP-2 are the only ELR$^+$CXC chemokines that chemoattract and activate neutrophils by binding to both CXCR1 and CXCR2, whereas the other ELR$^+$CXC chemokines only signal through CXCR2 (Wolf et al, 1998; Wuyts et al, 1998a). However, IL-8 is more potent than GCP-2 at activating neutrophils (Proost et al, 1993). Despite the weaker biologic potency of GCP-2 and the smaller amounts of GCP-2 secreted compared with those of IL-8, the selective up-regulation of GCP-2 production may be important in specific situations. In addition to the inducer dependency, the kinetics of chemokine production differed for GCP-2 and IL-8. For example, the IL-1β–induced GCP-2 concentration in fibroblasts increased until 144 hours after induction, whereas the IL-8 concentration remained constant from 24 hours onward. This indicates that, when IL-8 production is diminished, prolonged GCP-2 production might be important for attraction of neutrophils at later time points, eg, during chronic inflammation. In contrast, the dsRNA-induced IL-8 levels increased until 144 hours, whereas the GCP-2 concentration remained constant from 48 hours onward, suggesting a different role for these chemokines in viral infection. Furthermore, at later time points, LPS induced relatively more GCP-2 than IL-8 in fibroblasts, indicating that GCP-2 might be an important neutrophil-attracting and activating chemokine during bacterial infection. In addition to the different kinetics of GCP-2 and IL-8 production, it might be possible that specific GCP-2 isoforms have as yet unknown biologic activities, different from those of IL-8. Indeed, it has been shown that, in contrast to intact GROβ, a natural truncated isoform of this chemokine, missing four NH_2-terminal residues, has very potent hematopoietic activities in vitro and induces hematopoietic stem cell mobilization in vivo (King et al, 2000, 2001). This indicates that a particular chemokine or a chemokine isoform may, in addition to the shared leukocyte chemotactic and activating properties, have a unique function and hence play a predominant role in specific pathologies. Both IL-8 and ENA-78 have been detected in tissue specimens of patients with inflammatory bowel disease, but they may have different functions (Grimm et al, 1996; Mazzucchelli et al, 1994; Z'graggen et al, 1997). Indeed, ENA-78 was mainly expressed in epithelial cells, whereas the main cellular sources of IL-8 were inflammatory cells. It was suggested that ENA-78 plays a role in the onset of inflammatory bowel disease, whereas IL-8 may be more important in the perpetuation of the inflammation (Grimm et al, 1996; Mazzucchelli et al, 1994; Z'graggen et al, 1997). In this study, we have shown that GCP-2 protein is predominantly expressed in cells of the lamina propria of patients with Crohn's disease or colon carcinoma. Crohn's disease was chosen because this disease is an example of an inflammatory response in which bacterial LPS and cytokines are likely involved. In cattle, GCP-2 might have a specific role during pregnancy because GCP-2 is produced by the endometrium after stimulation with IFN-τ and pregnancy-specific protein B (Austin et al, 1999; Teixeira et al, 1997). Further measurements of GCP-2 concentrations in biologic fluids during disease will reveal whether GCP-2 is a prominent chemokine in a specific pathology.

In addition to its neutrophil-activating properties and in analogy with the other ELR$^+$CXC chemokines, GCP-2 stimulates angiogenesis through binding to CXCR2 (Addison et al, 2000; Strieter et al, 1995). As a result of the stimulation of angiogenesis, the ELR$^+$CXC chemokines have an important role in tumor development and metastasis. Elevated IL-8 and ENA-78 levels have been detected in human specimens of non-small cell lung cancer, and both chemokines contributed to tumor growth, tumor vascularity,

and metastasis (Arenberg et al, 1996, 1998). Furthermore, ELR$^+$CXC chemokines, produced by the tumor cells themselves or by surrounding tissues, may attract neutrophils toward tumors and activate these cells to release proteases, eg, gelatinase B, from their granules. Indeed, tumors selectively expressing GCP-2 were characterized by increased vascularization and by a strong influx and activation of neutrophils, leading to intratumoral secretion of gelatinase B (Van Coillie et al, 2001). Such attraction of neutrophils and release of proteases can favor tumor invasion and metastasis via the countercurrent principle (Opdenakker and Van Damme, 1992). Our study on the regulation of GCP-2 protein expression by tumor cells is thus relevant in tumor biology.

Taken together, our findings indicate that despite the apparent redundant activities of the neutrophil-attracting ELR$^+$CXC chemokines, their production is differently regulated in terms of inducer substances, production levels, tissue-specific expression, and kinetics of production. This indicates that these CXCR2-binding chemokines play different roles in physiologic and pathologic processes.

Materials and Methods

Production of Chemokines

For chemokine production on a large scale, confluent monolayers of MG-63 osteosarcoma cells were stimulated in Eagle's minimum essential medium with Earle's salts (EMEM; Invitrogen, Paisley, Scotland) supplemented with 2% FCS (BioWhittaker Europe, Verviers, Belgium) and a semipurified cytokine mixture for 48 hours at 37° C (Wuyts et al, 1997a). Alternatively, monocytes were purified from peripheral blood as previously described (Wuyts et al, 1999) and were stimulated with LPS (*Escherichia coli* 0.111.B4; Difco, Detroit, Michigan; 2 μg/ml) and conA (Calbiochem, La Jolla, California; 2 μg/ml) in EMEM supplemented with 2% FCS for 48 hours at 37° C. Chemokines produced on a large scale were isolated from conditioned cell supernatants by a four-step purification procedure as previously described (Wuyts et al, 1997a). Briefly, the conditioned media were first concentrated and partially purified by adsorption to controlled pore glass beads. The concentrated supernatants were subjected to antibody affinity chromatography or to heparin-Sepharose affinity chromatography. Finally, chemokines were purified to homogeneity by cation-exchange FPLC and RP-HPLC. Purified fractions were analyzed by SDS-PAGE under reducing conditions for purity and relative molecular mass. Pure proteins were identified by NH_2-terminal amino acid sequence analysis on a 477A/120A or a Procise 491 cLC protein sequencer (Applied Biosystems, Foster City, California).

For gene regulation experiments, human MG-63 osteosarcoma cells, Malavu hepatosarcoma cells, HeLa epithelial cervix carcinoma cells, IGROV ovarian carcinoma cells, and Bowes melanoma cells as well as human diploid fibroblasts (E_1SM or E_6SM, strains of embryonic skin and muscle cells) were grown to confluency in 24-well plates (1.9 cm^2; Techno Plastic Products AG, Trasadingen, Switzerland) or in 25-cm^2 flasks (Techno Plastic Products AG) in EMEM supplemented with 10% FCS. For induction, the growth medium was replaced by EMEM (1 ml/well or 5 ml/flask) supplemented with 0.5% or 2% FCS and with different concentrations of the following inducers: PMA (Sigma, St. Louis, Missouri), conA, LPS, measles virus (Attenuvax strain, $10^{6.5}$ 50% tissue culture infectious doses/ml), poly rI:rC (P-L Biochemicals, Milwaukee, Wisconsin), recombinant human IFN-γ (Bioferon, Laupheim, Germany), recombinant human TNF-α (Innogenetics Inc., Gent, Belgium), recombinant murine TNF-α (Theys et al, 1999), or pure natural human IL-1β (Van Damme et al, 1988). Supernatants were harvested after 48 hours and stored at $-20°$ C until assay. For kinetic experiments, inductions were always performed in 25-cm^2 flasks. Samples of 200 μl were taken at different time points and were replaced by 200 μl of medium.

Human umbilical vein endothelial cells were purchased from Clonetics (Walkersville, Maryland) and cultured following the manufacturer's instructions. Confluent monolayers were stimulated for 48 hours with different concentrations of the above-mentioned inducers in growth medium supplemented with 2% FCS. Chondrocytes from human articular cartilage were isolated and cultured as described previously (Van Damme et al, 1990) and stimulated for 24 hours in RPMI 1640 medium (BioWhittaker) supplemented with 10% FCS and different inducers. Pulmonary macrophages (kindly supplied by Dr. S. Dimova, Laboratory of Pneumology, University of Leuven) were isolated from airway tissues of four patients undergoing surgical resection for pulmonary carcinoma. These specimens were obtained in accordance with procedures approved by the ethical committee of the university. Tumor-free lung tissue was dissected and sliced, and the chopped tissue was rinsed by vigorous shaking with saline. Pulmonary macrophages were isolated from this washing fluid (Fathi et al, 2001; Hoet et al, 1999). Although the peripheral lung tissue was not normal senso stricto, it was distinguishable from the tumor tissue and was probably not different from that of a healthy person with similar smoking habits. Lung macrophages were induced at 10^6 cells per milliliter in EMEM containing 5% FCS.

Mononuclear cells and granulocytes were isolated from peripheral blood of single donors as described previously (Wuyts et al, 1997a). Peripheral blood mononuclear cells (5×10^6 cells per milliliter; serum free; $<$20% monocytes), blood monocyte-derived macrophages (after 5 days of culture of adherent monocytes in EMEM containing 5% FCS to allow maturation; $>$90% monocytes), or granulocytes (5×10^6 cells per milliliter; 0.5% FCS) were stimulated for chemokine production for 48 or 72 hours in EMEM supplemented with the above-mentioned inducers.

Measurement of Human IL-8, ENA-78, and GCP-2 by ELISA

For the measurement of human GCP-2, a classical sandwich ELISA was developed. Plates were coated (overnight, 4° C) with rabbit polyclonal anti-human GCP-2 antibody (1:500 dilution; PeproTech, Rocky Hill, New Jersey) in PBS. Remaining binding sites were blocked for 1 hour (37° C) with PBS containing 0.05% Tween 20 and 0.1% casein. Synthetic GCP-2 (Wuyts et al, 1997b) was used as a standard (0.002-2 ng/ml). Samples and standard were diluted in PBS containing 0.05% Tween 20 and 0.1% casein and transferred to the coated plate, incubated at 37° C for 1 hour, and subsequently washed with PBS with 0.05% Tween 20. Then, mouse monoclonal anti-human GCP-2 (1:2000 dilution; R&D Systems, Abingdon, United Kingdom) was added as second antibody (incubation for 1 hour at 37° C). After washing, the detection was performed with peroxidase-labeled goat anti-mouse antibody (1:5000 dilution; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) and 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB; Aldrich Chemical, Milwaukee, Wisconsin).

Human ENA-78 and IL-8 immunoreactivities were measured according to a similar procedure. Human ENA-78 was detected using a specific ELISA, recognizing both NH_2 terminally intact and truncated forms of ENA-78. Rabbit polyclonal anti-human ENA-78 (1:300) from PeproTech and goat polyclonal anti-human ENA-78 (1:300) from R&D Systems, respectively, were used as coating and capturing antibodies. Human IL-8 was measured with a classical sandwich ELISA using polyclonal goat anti-human IL-8 as coating antibody (1:800) and monoclonal mouse anti-human IL-8 ($1:10^4$) (R&D Systems) as capturing antibody, as described previously (Wuyts et al, 1999).

Detection limits for IL-8, ENA-78, and GCP-2 were 25, 200, and 8 pg/ml, respectively. The ELISAs were specific in that no other chemokines or chemokine inducers were detectable. CCL7 and CCL8 were chemically synthesized (Proost et al, 1995). Recombinant CXCL5, CXCL10, CCL3, CCL4, CCL5, and CCL17 were purchased from PeproTech; CCL20 was purchased from R&D Systems; and recombinant CXCL5 was purchased both from PeproTech (74 amino acids) and R&D Systems (78 amino acids).

Patients

Biopsy specimens of the intestinal wall were obtained from 10 patients with Crohn's disease and 4 patients with colon carcinoma. The diagnosis of Crohn's disease was based upon classical clinical, endoscopic, radiologic, and histologic criteria. The group of patients with Crohn's disease was composed of three men and seven women (mean age, 34 years), whereas the colon cancer group was composed of four men (mean age, 56 years). All Crohn's disease patients had active disease assessed with clinical and microscopic indices (mean microscopic score of 12.3 according to the scoring system of D'Haens et al, 1998).

Immunohistochemistry

Biopsy specimens were obtained from macroscopically involved and noninvolved intestinal areas for Crohn's disease patients and from normal colon for colon carcinoma patients. Cryostat sections (5 μm) were cut from freshly snap-frozen transmural biopsy samples, dried overnight, and fixed as previously described (Abu El-Asrar et al, 2000). Endogenous peroxidase activity was blocked for 5 minutes by treating the slides with 2% hydrogen peroxide in methanol. Then, slides were incubated for 3 hours with rabbit anti-human GCP-2 polyclonal antiserum (PeproTech; dilution 1:50). After incubation with peroxidase-labeled anti-rabbit antiserum (EnVision+; Dako, Carpinteria, California), the reaction product was visualized by 3-amino-9-ethylcarbazole (AEC), resulting in bright-red immunoreactive sites. The slides were faintly counterstained with Mayer's hematoxylin. As a negative control, frozen sections were treated according to the same procedure with the omission of the primary antibody.

Double immunohistochemical staining was performed with mouse antibodies against the monocyte/macrophage marker HLA-DR (1:50 dilution) (BD Biosciences, Erembodegem, Belgium). After staining for GCP-2 (without counterstaining), the slides were incubated for 30 minutes with the HLA-DR antiserum. Subsequently the sections were covered with alkaline phosphatase-conjugated anti-mouse antiserum (EnVision; Dako) for 30 minutes and colored with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Dako) for 30 minutes, giving a black color.

All sections, including those from macroscopically involved and noninvolved areas of Crohn's disease, were coded and scored blindly without prior knowledge of the origin of the sample. Cells staining positive for GCP-2 were counted in 10 different high-powered fields (×500 magnification, immersion) per biopsy specimen.

Statistical Analysis

Statistical analysis was performed using the Mann-Whitney *U* test.

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