gro-β, a -C-X-C- Chemokine, Is an Angiogenesis Inhibitor That Suppresses the Growth of Lewis Lung Carcinoma in Mice

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Summary

We have found that two chemokines, recombinant gro-α and gro-β, specifically inhibit growth factor–stimulated proliferation of capillary endothelial cells in a dose-dependent manner, whereas gro-γ has no inhibitory effect. In vivo, gro-β inhibits blood vessel formation in the chicken chorioallantoic membrane assay. It is sufficiently potent to effectively suppress basic fibroblast growth factor–induced corneal neovascularization after systemic administration in mice. Further, gro-β significantly inhibits the growth of murine Lewis lung carcinoma in syngeneic C57Bl6/J and immunodeficient nude mice without toxicity. In vitro, Lewis lung carcinoma cells are completely insensitive to recombinant gro-β at high concentrations that significantly inhibit endothelial cell proliferation. This finding supports the conclusion that gro-β inhibits Lewis lung tumor growth by suppression of tumor-induced neovascularization.

Angiogenesis, the formation of new capillaries from preexisting blood vessels, is a fundamental process involved in embryonic development and in pathological conditions such as tumorigenesis, diabetic retinopathy, and wound healing (1). The process of angiogenesis in vivo is regulated both by angiogenic stimulators such as fibroblast growth factors (FGFs)[1] and vascular endothelial growth factor (VEGF) (2, 3), and by angiogenic inhibitors such as thrombospondin and angiostatin (4, 5). Tumor growth is an angiogenesis-dependent process that requires the stimulation of new vessel growth (6). Tumors may locally generate an imbalance of angiogenic stimulation over inhibition by increasing the expression of angiogenic inducers or by decreasing the production of angiogenic suppressors. The identification of endogenous angiogenesis inhibitors may be useful for treatment of cancer and diabetic retinopathy.

Chemokines are members of a large superfamily of structurally and functionally related inflammatory cytokines that stimulate the chemotactic migration of distinct sets of cells, including neutrophils, monocytes, lymphocytes, and fibroblasts. gro-α, gro-β, gro-γ are members of a superfamily of small (8–10-kD), inducible, and secreted proinflammatory cytokines that all share four conserved cysteines (7). Two subfamilies can be distinguished according to the positions of the first two cysteines, which are either separated by one amino acid (-C-X-C- family) or are adjacent to each other (-C-C- family). Although each subfamily of chemokines has a common chemoattractant function for leukocytes and has a unique chromosomal location of genes (chromosome 4 for -C-X-C- proteins and chromosome 17 for -C-C- proteins), little is known about other distinct functions of the members of each family. The recent characterization of two -C-X-C- chemokines as angiogenic regulators (PF-4 as an angiogenic inhibitor and IL-8 as an angiogenic stimulator) (8–10) may help us to understand the physiological functions of the gro chemokines in the control of angiogenesis in vivo. In the present study, we investigated the antiangiogenic activities of gro chemokines by analyzing their effects on endothelial cells in vitro and in vivo, and on tumor growth in vivo.

Materials and Methods

Reagents, Cells, and Animals. Recombinant human gro-α, gro-β, gro-γ, and IL-8 expressed from *Escherichia coli* were purified by HPLC-coupled chromatography (R&D Systems Inc., Minneapolis, MN). Recombinant PF-4 was a generous gift of Dr. Theodore E. Maione (Repligen Corp., Cambridge, MA). Bovine capillary endothelial (BCE) cells were obtained as previously described (11) and were maintained in DME with 10% heat-inactivated bovine calf serum, antibiotics, and 3 ng/ml recombinant human basic FGF (bFGF) (Scios Nova Inc., Mountainview, CA). The Lewis lung tumor cell line was maintained in culture in DME supplemented with 10% FCS (Hyclone Laboratories, Logan, UT) and antibiotics. Male 6–8-wk-old C57Bl6/J mice (The Jackson Laboratory, Bar Harbor, ME) and nude mice (Massachusetts General Hospital, Boston, MA) were used for tumor studies. Animals were

[1] *Abbreviations used in this paper:* BCE, bovine capillary endothelial; bFGF, basic fibroblast growth factor; CAM, chick chorioallantoic membrane; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor.

anesthetized in a methoxyflurane (Pitman-Moore, Inc., Mundelein, IL) chamber before all procedures and were observed until fully recovered. Animal studies were reviewed and approved by the animal care and use committee of Children's Hospital (Boston, MA) and are in accordance with the guidelines of the Department of Health and Human Services (U.S. Department of Agriculture).

Iodination Labeling. For iodination labeling, 5 μg of recombinant human gro-β and 5 μg IODO-GEN (Pierce, Rockford, IL) were dissolved in chloroform in a final volume of 100 μl. The gro-β and IODO-GEN mixture was dried in a glass tube by evaporating the reagent with dried nitrogen gas. The reagents were dissolved in 0.2 M phosphate buffer, pH 7.2. Na ^{125}I (New England Nuclear, Boston, MA) (500 μCi) was added to the reaction and incubated 15 min at room temperature. The reaction was terminated by addition of 50 μl of 10 mM KCl, 10% TCA, and 1 mg/ml BSA. Labeled protein was separated from unincorporated reagents on Sephadex G-25 (Pharmacia Biotech, Inc., Piscataway, NJ). Specific activity of the ^{125}I–gro-β was 15 cpm pg^{-1}.

Heparin-binding Chromatography. Approximately 10 μg of purified gro-α, gro-β, gro-γ, and PF-4 was loaded onto a heparin TSK™-gel column (7.5 mm × 7.5 cm) in a phosphate buffer (20 mM sodium phosphate, pH 7.5). The proteins were eluted with a linear gradient of NaCl in 20 mM sodium phosphate buffer, pH 7.5), at a rate of 1 ml/min. Fractions of 1 ml were collected and measured for protein concentrations at 280 nm.

DNA Synthesis Assay for Capillary Endothelial Cells. BCE cells were seeded in 48-well plates (10,000 cells per well) and grown in DME supplemented with 10% bovine calf serum. After 24 h, the medium was replaced with fresh DME (GIBCO BRL, Gaithersburg, MD) containing 2% bovine calf serum, 1 mM thymidine, and 0.5% BSA. The cells were incubated in 10% CO_2 at 37°C for 48 h, and samples (recombinant gro-α, gro-β, gro-γ, and PF-4) at different concentrations were added to the cells in the presence of 1 ng/ml bFGF. After 16 h of incubation, 0.5 μCi of [^{3}H]thymidine in 10 μl PBS was added to each well. 5 h later, the medium was aspirated and the cells were washed twice with PBS. The cells were incubated on ice with two 5-min methanol and two 10-min 5% TCA incubations. The fixed cells were washed once with distilled water and solubilized in 200 μl of 0.3-M NaOH. The incorporated radioactivity was determined in a scintillation counter (Wallac, Gaithersburg, MD).

Proliferation Assay for Capillary Endothelial Cells and Tumor Cells. A 72-h BCE cell proliferation assay was performed as previously described (7). For the tumor cell proliferation assay, Lewis lung carcinoma cells (8,000 cells per well) were plated onto 24-well tissue culture plates and were incubated in DME medium (0.5 ml/well) containing 5% FCS and antibiotics for 24 h, and each sample in triplicate was added to cells. After 72 h, adherent and nonadherent cells were dispersed in trypsin, resuspended in Hematall (Fisher Scientific Co., Pittsburgh, PA), and counted by Coulter counter.

Chick Chorioallantoic Membrane (CAM) Assay. 3-d-old fertilized white Leghorn eggs (Spafas, Inc., Norwich, CT) were cracked, and chick embryos with intact yolks were placed in 100 × 20 mm plastic petri dishes. After 3 d of incubation in 3% CO_2 at 37°C, a disk of methylcellulose containing a chemokine protein was implanted on the CAM of individual embryos. The disks were made by desiccation of 10 μl of 45% methylcellulose (in H_2O). After 48 h of incubation, embryos and CAMs were analyzed for the formation of avascular zones by a stereomicroscope.

Mouse Corneal Micropocket Assay. 6-wk-old male C57Bl6/J mice, each ~20 g, were divided into three groups. Control animals (n = 9) received daily intraperitoneal injections of 0.2–0.3 ml PBS. One group of animals (n = 5) received daily intraperitoneal injections of 15 mg/kg gro-β in 0.2 ml PBS, including pretreatment with the same dose of gro-β for 2 d before corneal implantation. The third group of animals (n = 3) received daily intraperitoneal injections of 100 mg/kg gro-β in 0.3 ml PBS beginning on the day of corneal implantation. A corneal micropocket was created in both eyes of each mouse as previously described (7). Into this pocket, pellets containing sucrose aluminum sulfate (Bukh Meditec, Copenhagen, Denmark), hydron polymer type NCC (Interferon Sciences, Inc., New Brunswick, NJ), and 80–100 ng of bFGF were implanted as previously described (7). The corneas of all mice were photographed by means of a slit-lamp stereomicroscope at a magnification of 10 on the sixth day after corneal implantation. Maximal vessel length and clock hours of circumferential neovascularization were measured.

Histology. Animals were killed on day 6 after corneal implantation, and eyes were enucleated and fixed in 10% phosphate-buffered formalin. Eyes were embedded in paraffin according to standard histological procedures. Cross-sections of the eye (5 μm thick) were permeabilized with 0.2 M HCl for 10 min. The sections were processed and stained with a rabbit antiserum against human von Willebrand factor (Dako Corp., Carpinteria, CA) as previously described.

Tumor Studies in Mice. Male 6–8-wk-old C57Bl6/J and immunodeficient nude mice were used for tumor studies. Lewis lung carcinoma cells (1 × 10^6) growing in log phase were harvested, resuspended in PBS, and implanted subcutaneously in the midline dorsum of each animal in a volume of 100 μl. Four mice were used in each treatment or control group. Intralesional injections with either 100 μl PBS or 100 μg of gro-β in 100 μl PBS were begun shortly after injection of tumor cells and continued once daily for a total of 14–15 treatments. Visible tumors were present within 24 h. Primary tumors were measured blindly using digital calipers on the days indicated.

Results and Discussion

Amino Acid Sequence Alignment and Heparin-binding Affinity of Human Chemokines. The overall sequence identity and consensus amino acids were obtained by comparing gro-α, gro-β, gro-γ, PF-4, and IL-8 after removal of NH_2-terminal signal sequences (Fig. 1 *a*). The gro proteins share high sequence identity with each other (86–90%). In the COOH-terminal region, they are homologous with PF-4 (54% identity), including a shared cluster of positively charged lysines (Fig. 1 *b*), whereas they are less homologous to IL-8 (15% identity) in this region (Fig. 1 *a*). Recombinant human -C-X-C- chemokines (PF-4, IL-8, gro-α, gro-β, and gro-γ) expressed from *E. coli*, were purified to >95% homogeneity as revealed by SDS gel analysis (Fig. 1 *c*). The gro-β protein in vitro at a concentration of 0.5 mg/ml is in equilibrium between monomeric and dimeric forms (Fig. 1 *d*), suggesting that these chemokines with a similar backbone structure may be active in either form (12). The gro proteins are heparin-binding molecules with a similar affinity as evidenced by elution from a heparin-Sepharose column with 0.5 M NaCl. PF-4, which eluted from an identical column with 1.0 M NaCl, has a higher affinity for heparin (Fig. 1 *e*).

Inhibition of DNA Synthesis of Endothelial Cells by gro Chemokines. To determine whether the gro family could inhibit the proliferation of endothelial cells in vitro, recombinant

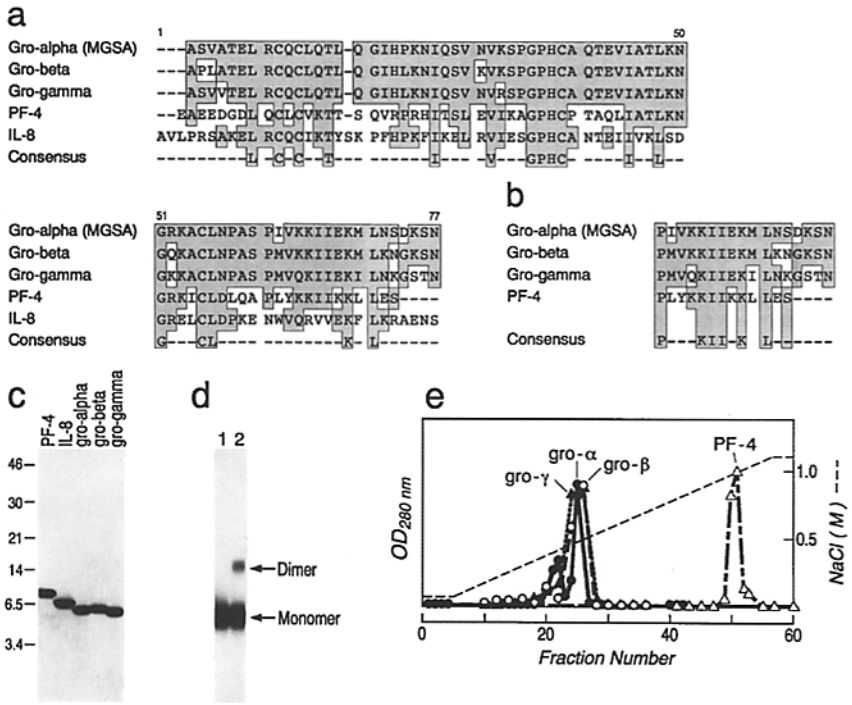


Figure 1. Amino acid sequence alignment and biochemical characterization of human chemokines. (*a* and *b*) The sequences for five -C-X-C- chemokines (gro-α, gro-β, gro-γ, PF-4, and IL-8) are aligned according to their conserved cysteines. Identical and conserved amino acids are boxed and shaded. (*c*) SDS-PAGE analysis of purified recombinant chemokines under reducing conditions. 5 μg of each protein was loaded onto a 15% SDS gel followed by staining with Coomassie blue. (*d*) Chemical cross-linking of ^{125}I–recombinant human gro-β. The labeling was performed as described in Materials and Methods. Lane *1*, ^{125}I–gro-β without disuccinimidyl suberate cross-linker. Lane *2*, ^{125}I–gro-β (0.5 mg/ml) incubated with disuccinimidyl suberate at room temperature for 30 min. 5 ng of each sample was analyzed by SDS-PAGE. (*e*) Elution profile of purified gro-α, gro-β, gro-γ, and PF-4 from a heparin TSK-gel column.

human gro proteins were incubated with BCE cells (11) stimulated by bFGF. At the concentration of 10 μM, gro-α and gro-β each inhibited DNA synthesis by 56%, which was less potent than recombinant PF-4 (76% inhibition) (Fig. 2 *a*). The dose-dependent inhibition of DNA synthesis of BCE cells by gro-β correlates with the inhibition of cell proliferation (Fig. 2 *c*). No distinct morphology associated with apoptotic endothelial cells such as rounding, detachment, and fragmentation of cells could be detected, even after a 3-d incubation with gro-β (Cao, Y., unpublished data). Recombinant gro-γ, however, which shares 87 and 86% amino acid sequence identities with gro-α and gro-β, respectively (Fig. 1 *a*), had no inhibitory activity on bovine capillary endothelial cells (Fig. 2, *a* and *b*). Previous studies reported that the inhibition of angiogenesis by PF-4 could be reproduced by a synthetic peptide derived from the COOH-terminal 13 amino acids of PF-4, which contains a cluster of positively charged lysines (8). Although all three gro proteins share high degrees of sequence homology with PF-4 in the COOH-terminal region, gro-γ has one amino acid substitution (from lysine to glutamine) at position 64 in the conserved lysine-enriched region (Fig. 1, *a* and *b*). The same lysine to glutamine substitution at position 64 is also found in IL-8 (Fig. 1 *a*). In contrast to PF-4, IL-8 has been reported to be a potent angiogenic factor (10, 13). Thus, the substitution of a lysine residue by glutamine in the COOH terminus may contribute to the lack of endothelial cell inhibitory activity of gro-γ and IL-8.

Unlike PF-4, the inhibitory activities of gro-α and -β on BCE cells could not be abrogated by addition of soluble

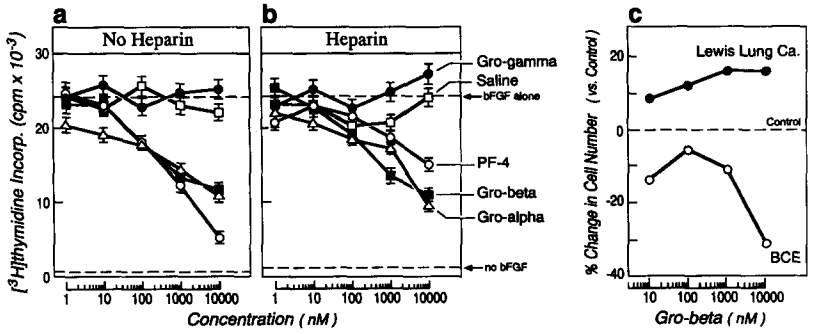


Figure 2. Inhibition of DNA synthesis and proliferation of capillary endothelial cells by chemokines (*a* and *b*) BCE cells were seeded in 48-well plates (10,000 cells per well) and grown in DME supplemented with 10% BCS as described in Materials and Methods. Recombinant chemokines at different concentrations were added to cells containing 1 ng/ml bFGF in the presence and absence of heparin (10 U/ml). 24 h later, [^{3}H]thymidine was added to each well. Radioactivity was measured 5 h later, (*a*) in the absence of heparin, (*b*) in the presence of 10 U/ml heparin. ○, PF-4; □, bFGF alone; △, gro-α; ■, gro-β; ●, gro-γ. (*c*) Recombinant human gro-β at different concentrations was tested on BCE cells in the presence of 1 ng/ml bFGF in a 72-h proliferation assay as described in Materials and Methods. gro-β inhibited capillary endothelial cell proliferation in a dose-dependent manner (○). Recombinant gro-β at doses up to 10 μM did not inhibit proliferation of murine Lewis lung carcinoma cells (●) in vitro. Values represent the mean of three determinations (± SEM) as percentages of inhibition or stimulation.

heparin (10 U/ml; Sigma Chemical Co., St. Louis, MO) to the culture medium (Fig. 2 *b*). In contrast, the antiproliferative activity of PF-4 was largely abolished by heparin (Fig. 2 *b*). Previous reports have suggested that PF-4 may block the low-affinity binding sites of bFGF composed of heparan sulfate proteoglycans and thereby inhibit angiogenesis induced by bFGF (14, 15). Although gro chemokines are heparin-binding proteins, their affinity for heparin (0.5 M NaCl elution) appears to be too low to interfere with binding of bFGF to heparan sulfate proteoglycans on the cell surface and in the extracellular matrix. Further, the COOH-terminal 13 amino acids of and a mutant analogue derived from PF-4, both do not bind to heparin yet inhibit angiogenesis (9). These findings argue against blockage of heparan sulfate proteoglycan-binding sites for bFGF as a mechanism for the angiostatic effects of these chemokines. Whether the inhibition of angiogenesis by these chemokines is mediated by a specific receptor(s) expressed on endothelial cells or by other alternative mechanisms must be determined. Our preliminary results obtained by cross-linking ^{125}I–gro-β, and ^{125}I–PF-4 to bovine capillary endothelial cells indicate that no specific high-affinity receptors for these chemokines are present on endothelial cells (Cao. Y., unpublished data).

Inhibition of Angiogenesis in the Chick Embryo by gro-β. To study the angiostatic activity in vivo, recombinant gro proteins were tested on the CAM (16, 17). At a concentration of 10 μg/disk, gro-β inhibited new vessel growth in all chick embryos tested as measured by the formation of avascular zones (Fig. 3, *a* and *c*). Vessel regression around the avascular zone was seen in the chick membranes implanted with gro-β disks (Fig. 3, *a*, *inset*). The measured inhibition of gro-β was dose dependent, and no detectable inflammation was observed. gro-α and gro-γ had moderate inhibitory effects on new vessel growth in this assay. These results confirm our in vitro data that gro-β is a specific inhibitor for endothelial cells.

Inhibition of Mouse Corneal Neovascularization by gro-β. To further investigate the antiangiogenic activity of gro-β in vivo, the effect of systemic administration of gro-β on bFGF-stimulated neovascularization in the mouse cornea was studied. This bioassay requires that a putative angiogenesis inhibitor be administered systemically (e.g., intraperitoneal injection) to test its ability to suppress bFGF-induced neovascularization in the cornea. This is a rigorous assay that can discriminate between the many substances inhibiting angiogenesis in the chick embryo, or in in vitro assays, and those few compounds that are sufficiently potent to inhibit angiogenesis in remote sites such as the cornea when induced by a relatively high dose of 80 ng bFGF. Systemic treatment with gro-β inhibited bFGF-stimulated corneal neovascularization in a dose-dependent manner (Fig. 4). The length of growing vessels was inhibited in a dose-dependent manner by intraperitoneal injections of gro-β from 15 mg/kg ($P < 0.002$) up to 100 mg/kg ($P < 0.001$), (inhibition of length of ~40%). There was no significant difference in clock hours of circumferential neovascularization in treated animals compared with control animals (Fig. 4 *f*). The density of new vessels in the gro-β–treated animals was also markedly reduced as compared with that of control animals (Fig. 4, *a* and *b*). The treated mice did not experience weight loss over the course of treatment, indicating that no toxicity was caused by the pharmacological dose of gro-β.

Since gro-β acts as a chemoattractant for neutrophils, the observed inhibition of vessel growth may be due to an indirect effect mediated by these inflammatory cells. However, histological examination of corneas implanted with bFGF pellets from gro-β–treated animals and control animals revealed no significant differences in the number of inflammatory cells within the corneal stroma (Fig. 4, *c* and *d*). In the control animals, vessels stained by an antibody against von Willebrand factor (18) can be seen within the corneal stroma adjacent to the implanted FGF pellet (Fig. 4 *c*). No vessels are seen in the corneal section from gro-β–treated animals (Fig. 4 *d*). These findings support the data obtained from the in vitro endothelial cell proliferation assay and the CAM assay that gro-β has direct inhibitory effects on new vessel growth.

Reversible Inhibition of Primary Tumor Growth by gro-β In Vivo. To determine if gro-β could inhibit primary tumor growth, recombinant gro-β protein was used to treat C57Bl6/J mice bearing subcutaneous Lewis lung carcinomas. Daily intralesional injections of 100 μg of gro-β per 20 g mouse completely suppressed the growth of primary tumors during the 14-d treatment course (Fig. 5 *a*). In contrast, tumors grew rapidly to sizes >200 mm^3 in all saline-treated animals during the same 14-d treatment period. Upon termination of the intralesional injections of gro-β, regrowth of tumors was observed in 75% of the gro-β–treated animals within 6 wk after tumor implantation (Fig. 5 *b*). Tumors in animals treated with saline continued to grow exponentially to >7,000 mm^3, leading to the demise of all animals on day 40 after tumor implantation. To exclude the possibility that an immune response induced by gro-β was involved in its antitumor activity, immunodeficient nude mice were also used to determine the antitumor effect of gro-β. As shown in Fig. 5 *c*, the growth of Lewis lung carcinoma in nude mice was also significantly inhibited by intralesional injection of gro-β although the efficacy of inhibition (~60%) was not as potent as that observed in immunocompetent mice (Fig. 5, *a* and *b*). These data support the notion that suppression of Lewis lung tumor growth by gro-β is mediated by inhibition of tumor-induced angiogenesis. It would be interesting to investigate if gro-β can also suppress the growth of other tumors.

Inhibition of Cell Proliferation by gro-β Is Specific for Endothelial Cells but Not for Tumor Cells. To further exclude the possibility that the antitumor activity of gro-β was due to direct effects on tumor cells, gro-β was tested in vitro on Lewis lung carcinoma cells and endothelial cells in a 72-h proliferation assay. At a concentration of 10 μM gro-β, the proliferation of BCE cells was significantly inhibited by ~40% (Fig. 2 *c*). The inhibition of endothelial cell proliferation occurred in a dose-dependent manner, whereas proliferation of Lewis lung carcinoma cells was not affected at all doses used. These results are consistent with the inhibitory effects of gro-β on DNA synthesis of endothelial cells

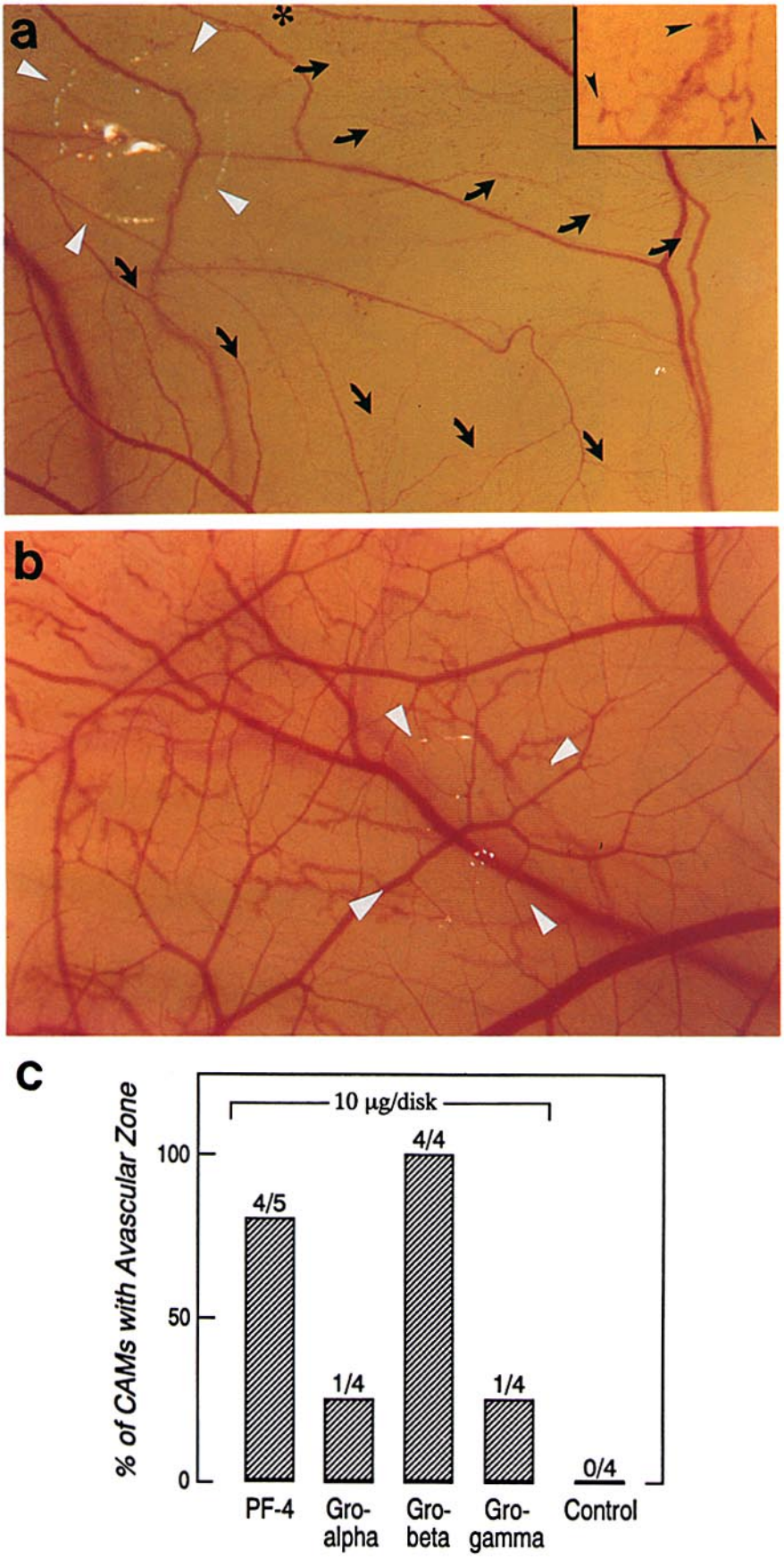
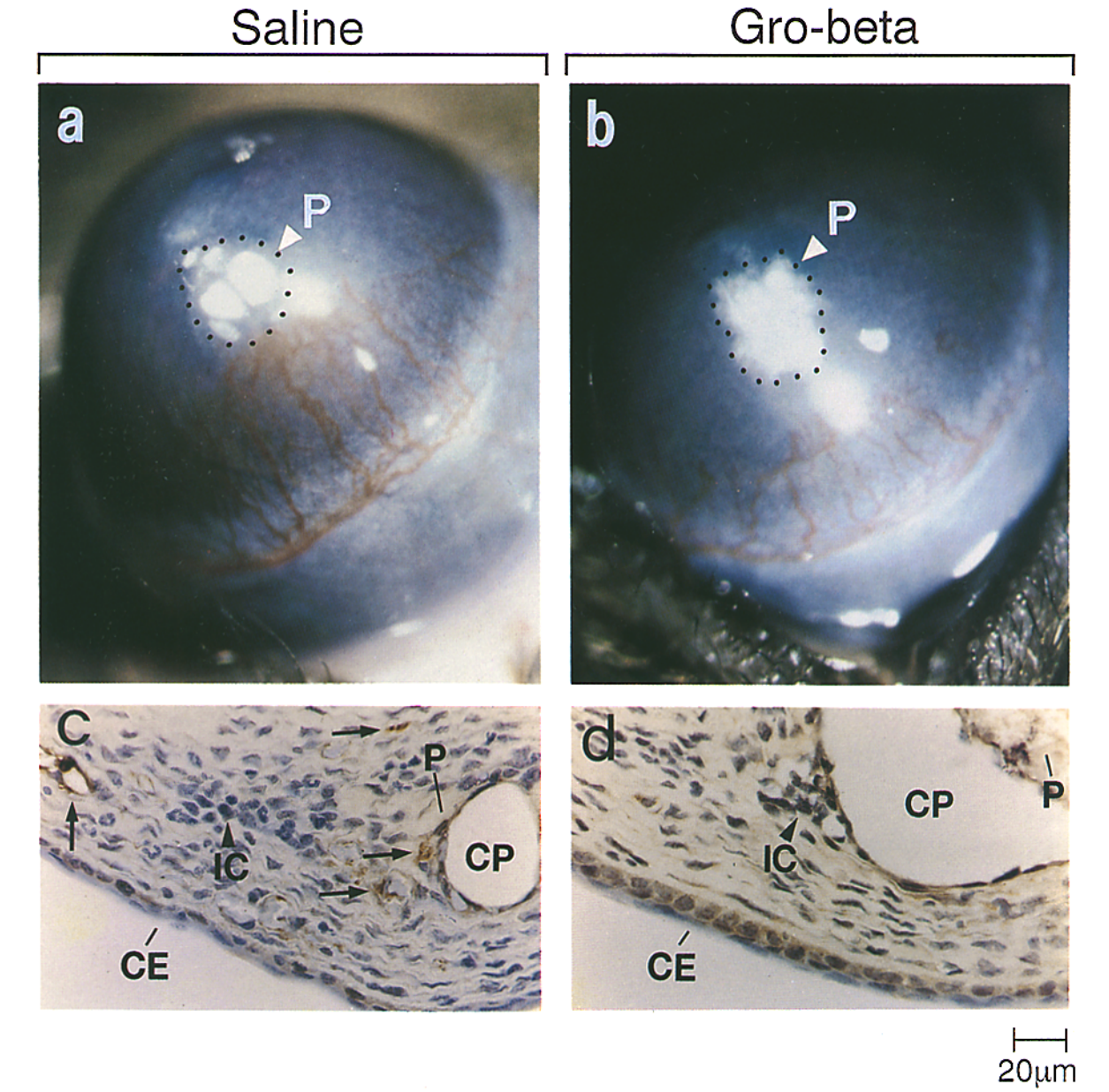


Figure 3. Antiangiogenic effects of gro-α, gro-β, gro-γ, and PF-4 on the CAM. Methylcellulose disks containing 10 μg of each protein were implanted on CAMs of 6-d-old chick embryos as described. After 48 h, the formation of avascular zones was analyzed. (*a*) An example of gro-β–implanted CAM. The area of the avascular zone is marked by curved arrows. White arrowheads point to the implanted disk (2-mm diameter). High magnification insert of the area marked by an asterisk reveals the regressed vessels (*arrows*) in the adjacent area of the avascular zone. (*b*) A control CAM with methylcellulose disk (*arrowheads*) containing saline. (*c*) At a concentration of 10 μg/disk, the number of CAMs with avascular zones over the total number of CAMs tested for each protein is indicated above each bar.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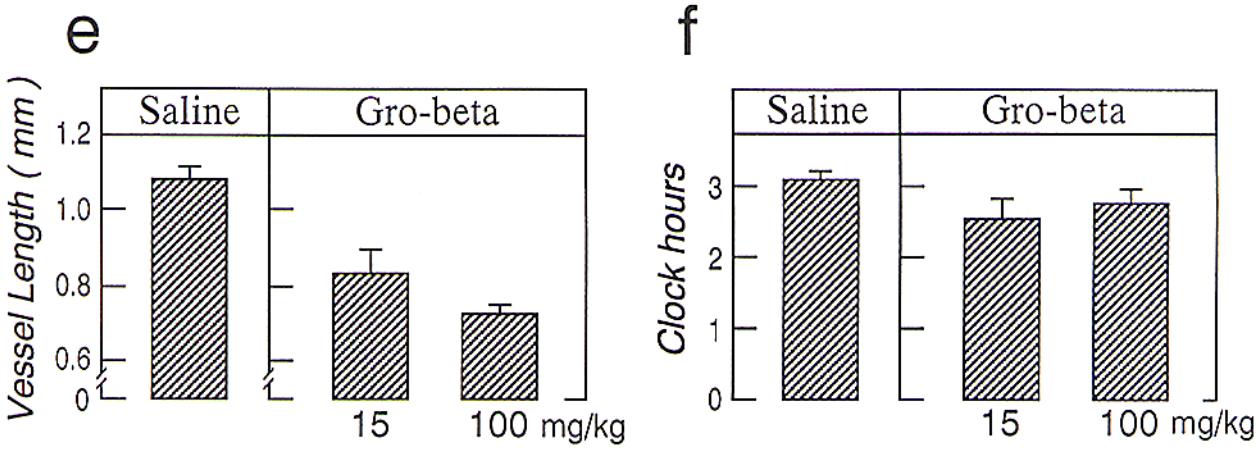
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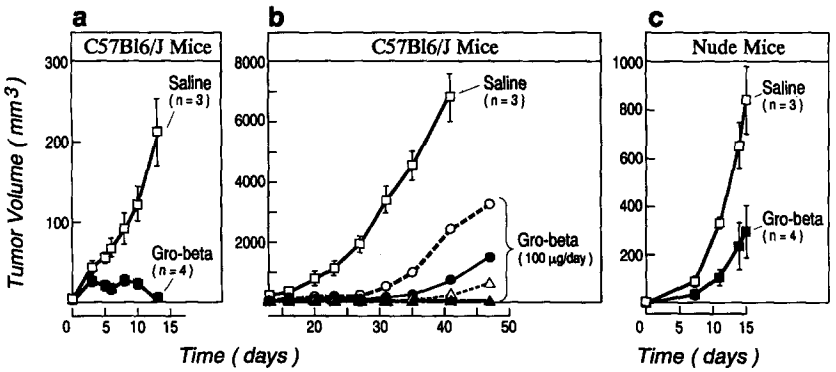

Figure 5. Inhibition of the growth of Lewis lung carcinoma in syngeneic C57Bl6/J and immunodeficient nude mice by recombinant human gro-β. (*a*) Recombinant gro-β (■) and PBS (□) were tested for their effects on subcutaneous tumor growth in C57Bl6/J mice. gro-β protein at a final concentration of 1.0 mg/ml was injected intralesionally in volume of 100 μl per mouse shortly after the implantation of tumor cells once daily through day 13. Primary tumors were measured blindly using digital calipers on the days indicated. For days 0–13, data represent the mean tumor volumes (± SEM) of surviving mice in each group. (*b*) Treatments were discontinued after day 13, and primary tumor volumes were measured as above. 40 d after tumor implantation, all animals in the control group died. For days 13–40, data for the saline-treated animals represent the mean tumor volumes (± SEM) of surviving mice (□), whereas data for the gro-β–treated animals are represented individually for each animal from days 13–47 (○, ●, △, and ▲). (*c*) Recombinant gro-β (■) and PBS (□) were examined for their effects on subcutaneous tumor growth in nude mice. The intralesional treatments were carried out using the same procedures as described (*a*), except treatments were continued through day 14. For days 0–15, data represent the mean tumor volumes (± SEM) of surviving mice in each group.

(Fig. 2, *a* and *b*), suggesting that gro-β is an angiogenesis inhibitor with no direct effect on proliferation of Lewis lung tumor cells.

The -C-X-C- chemokines have previously been identified to be chemotactic for certain populations of leukocytes, such as neutrophils that are involved in the inflammatory response. The physiological functions of these proteins in vivo, however, remain to be characterized, since their in vitro activities do not always predict their predominant in vivo effects. To date, two members of the -C-X-C- family, IL-8 and PF-4, have been shown to be involved in the regulation of angiogenesis (8–10). IL-8 has been shown to be a potent stimulator of angiogenesis, and PF-4 has been found to be an inhibitor of angiogenesis. These opposing activities related to the regulation of angiogenesis by a family of structurally related proteins suggest the presence of multiple, yet divergent, biological functions of the chemokines. Recently, proliferin and proliferin-related protein, two members of the prolactin and growth hormone family, have been shown to have opposing regulatory effects on angiogenesis during placenta formation (19). It is interesting to speculate on the role of certain -C-X-C- chemokines during the inflammatory response. The up-regulation of an angiogenic chemokine such as IL-8 and the down-regulation of angiostatic chemokines such as PF-4 and gro-β may accelerate angiogenesis during wound healing. In the later phases of inflammation, the expression of angiogenic chemokines may be switched off while the expression of angiostatic chemokines are switched on. In this respect, it would be important to examine the expression levels of these chemokines during all phases of inflammation. Recently, Hoogewerf et al. (20) have discovered that the -C-X-C- chemokines, CTAP-III and NAP-2, are heparan sulfate–degrading enzymes. The degradation of heparan sulfate in the extracellular matrix and on the cell surface may play a key role in the regulation of angiogenesis, since angiogenic factors such as bFGF and VEGF exert their functions by interacting with both specific high affinity receptors and heparan sulfate proteoglycans on the cell surface.

Comparison of amino acid sequence homology reveals that, in the COOH-terminal region, gro chemokines share high sequence homologies with the 13 amino acids of PF-4 (Fig. 1, *a* and *b*), which has been reported to be an angiostatic peptide (8). Mutations of crucial amino acids in this region, such as a positively charged lysine in position 64 (conserved among PF-4, gro-α, and gro-β) to glutamine in gro-γ and IL-8 (Fig. 1 *a*), may lead to the loss of angiostatic activity. Since the genes for -C-X-C- chemokines are located in the same region (q21) of chromosome 4 because of gene duplication (7), the mutations caused by gene duplication during evolution may explain the divergence of biological activities of these chemokines.

An increasing number of endogenous angiogenic inhibitors, including the recently isolated angiostatin from our laboratory (5), have been identified in the last decade. It should be emphasized that gro-β is one of the few angiogenesis in-

Figure 4. Inhibition of mouse corneal neovascularization by systemic administration of gro-β. Pellets containing sucrose aluminum sulfate, hydron, and 80–100 ng of bFGF were implanted into corneal micropockets of mice as described in Materials and Methods. Corneas were photographed by slit-lamp stereomicroscopy on day 6 after bFGF pellet implantation. (*a*) Cornea of control mouse receiving daily intraperitoneal injections of saline. (*b*) Cornea of mouse treated with daily intraperitoneal injections of 100 mg/kg gro-β. Immunohistochemical staining of cross-sections of mouse corneas using antibodies to von Willebrand factor. *P*, the location of the implanted pellet. (*c*) Saline-treated animal. (*d*) Animal treated with intraperitoneal injection of 100 mg/kg gro-β administered as intraperitoneal injection. Arrows point to stained vessels within corneal stroma. *CE*, corneal epithelium; *CP*, corneal pocket; *IC*, inflammatory cells; *P*, pellet. Bar, 20 μm. Quantitation of corneal neovascularization was performed on day 6 after bFGF pellet implantation. (*e*) Maximal vessel length. (*f*) Clock hours of circumferential neovascularization.

hibitors that can effectively inhibit bFGF-induced neovascularization in the mouse cornea after systemic administration. The expression of two other endogenous endothelial cell inhibitors, thrombospondin and glioma-derived angiogenesis inhibitory factor, has been reported to be controlled by the tumor suppressor gene p53 (21, 22). The loss of function of the p53 gene leads to the down-regulation of these two angiogenic inhibitors and may thereby promote tumor growth. Whether other endogenous angiogenesis inhibitors are controlled by tumor suppressor genes such as p53 remains to be seen.

In the present study, we also show that gro-β is a potent inhibitor of Lewis lung tumor growth in both immunocompetent and immunodeficient mice without direct inhibitory activity on tumor cells. Like angiostatin and TNP-1470, two known inhibitors of angiogenesis, gro-β may allow tumor cells to remain dormant by inhibiting tumor cell–induced neovascularization (23, 24). We cannot exclude the possibility that other mechanisms may also be involved in the gro-β–mediated antitumor activity. Many -C-X-C- and -C-C- chemokines have recently been found to be potent antitumor peptides. For example, IP-10 in the -C-X-C- family (25) and JE/MCP-1 protein in the -C-C- family (26) inhibit the growth of plasmacytomas and tumor formation from transformed Chinese hamster cells, respectively. Although these chemokines may exert antitumor activities by attracting leukocytes such as monocytes and T lymphocytes, they, like PF-4 (27) and gro-β, may inhibit tumor growth via either direct or indirect antiangiogenesis pathways.

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Note added in proof: In a recent paper (Angiolillo, A. L., C. Sgadari, D. D. Traub, F. Liao, J. M. Farber, S. Maheshwari, H. K. Kleinman, G. H. Reaman, and G. Tosato. 1995. *J. Exp. Med.* 182:155–162.) reported that human IFN-inducible protein 10, another -C-X-C- chemokine, is a potent inhibitor of angiogenesis in vivo.

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