

Human Interferon-inducible Protein 10 Is a Potent Inhibitor of Angiogenesis In Vivo

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Summary

Human interferon-inducible protein 10 (IP-10), a member of the α chemokine family, inhibits bone marrow colony formation, has antitumor activity in vivo, is chemoattractant for human monocytes and T cells, and promotes T cell adhesion to endothelial cells. Here we report that IP-10 is a potent inhibitor of angiogenesis in vivo. IP-10 profoundly inhibited basic fibroblast growth factor-induced neovascularization of Matrigel (prepared by H. K. Kleinman) injected subcutaneously into athymic mice. In addition, IP-10, in a dose-dependent fashion, suppressed endothelial cell differentiation into tubular capillary structures in vitro. IP-10 had no effect on endothelial cell growth, attachment, and migration as assayed in vitro. These results document an important biological property of IP-10 and raise the possibility that IP-10 may participate in the regulation of angiogenesis during inflammation and tumorigenesis.

Angiogenesis, the process of generating new blood vessels leading to neovascularization, is essential during reproduction, embryonic development, tissue and organ growth, and wound healing (1). Unbalanced neovascularization is believed to contribute to the pathogenesis of certain disease states, such as arthritis, psoriasis, hemangiomas, diabetic retinopathy, and retrolental fibroplasia, and to allow tumor growth and metastasis to occur (1). Tumor cells must attract new vessels to expand locally and produce metastasis (1, 2). Thus, angiogenesis inhibitors might be developed into effective anticancer drugs.

Several compounds have been reported to inhibit endothelial cell proliferation in various experimental systems, including TGF- β (3), thrombospondin (4), IL-1 (5), IFN- γ and - α (6), tissue inhibitor of metalloproteinase 1 (7), platelet factor (PF)¹ 4 (8), protamine (9), and fumagillin (10). IFN- α and

a fumagillin derivative, AGM-1470, have reached clinical testing (2).

Recently, we have reported on an experimental athymic mouse model in which regression of human Burkitt's lymphoma is induced by intratumor inoculation of EBV-immortalized human B cells (11). Extensive central necrosis associated with endothelial cell damage and intravascular thrombosis often distal to the necrotic tumor tissue is typical of regressing tumors (11). This suggested that tissue ischemia may be central to tumor regression and raised the possibility that unbalanced angiogenesis might be responsible for regression of Burkitt's lymphoma in this system (11). Analysis of murine cytokine expression showed that IL-6, TNF- α , and interferon-inducible protein 10 (IP-10), but not other cytokines, are expressed at higher levels by regressing tumors compared with progressing tumors (11). Because the biological functions of IP-10 are incompletely understood, we have tested the possibility that IP-10 might function as an inhibitor of angiogenesis and might contribute to tumor regression.

Materials and Methods

Mice, Cells, Reagents, and Cytokines. 4–6-wk-old female BALB/c nu/nu mice (Charles River Laboratories, Wilmington, MA, or Na-

¹ Abbreviations used in this paper: bFGF, basic fibroblast growth factor; ECGS, endothelial cell growth supplement; FBHE, fetal bovine heart endothelial cells; HUVEC, human umbilical vein endothelial cells; IP-10, interferon-inducible protein 10; MCAF, macrophage chemotactic and activating factor; Mig, monokine induced by IFN- γ ; PF, platelet factor; RANTES, regulated on activation, normal T cell expressed and secreted.

tional Cancer Institute, National Institutes of Health, Frederick, MD) maintained in pathogen-limited conditions were used throughout. Matrigel was extracted by H. K. Kleinman from the Englebreth-Holm-Swarm tumor as previously described (12). Recombinant human IP-10 (0.06 endotoxin U/ μ g, Pepro Tech, Inc., Rocky Hill, NJ) was either purchased or provided by the National Cancer Institute. TGF- β was obtained from R&D Systems (Minneapolis, MN). The human monokine induced by IFN- γ (Mig) protein was the mature, 103-amino acid recombinant protein purified from an overexpressing Chinese hamster ovary cell line. The purification and characterization of Mig will be described elsewhere (Liao, F., R. Rabin, L. Koniaris, P. Vanguri, J. Yannelli, and J. Farber, manuscript in preparation). Mig concentration was determined using the Bradford assay (Bio-Rad Laboratories, Richmond, CA) standardized with BSA. Recombinant human IL-8 was obtained from BioSource, International (Camarillo, CA). Recombinant human macrophage chemotactic and activating factor (MCAF) and recombinant human regulated on activation, normal T cell expressed, and secreted (RANTES) were obtained from Pepro Tech, Inc., and recombinant human PF-4 was a gift of Dr. T. Maione (Repligen Co., Cambridge, MA). Fetal bovine heart endothelial cells (FBHE; American Type Culture Collection, Rockville, MD) were grown in DMEM (Biofluids, Inc., Rockville, MD) containing 10% heat-inactivated fetal bovine serum (Intergen Co., Purchase, NY), 100 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems), and 5 μ g/ml gentamicin (Sigma Chemical Co., St. Louis, MO). Human umbilical vein endothelial cells (HUVEC), from American Type Culture Collection or provided by the National Institute of Dental Research (National Institutes of Health), were maintained in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD), 15% FBS, 20 U/ml porcine heparin (Squibb-Marsam, Inc., Cherry Hill, NJ), and 100 μ g/ml endothelial cell growth supplement (ECGS; a crude extract of bovine neural tissue containing bFGF and acidic FGF, Calbiochem-Novabiochem Corp., La Jolla, CA). H5V, a murine heart endothelioma cell line, was a gift from Dr. A. Mantovani (Istituto di Ricerche Farmacologiche, Milan, Italy) (13). CD3-21, a murine pulmonary microvascular endothelial cell line, was kindly provided by Dr. C. Diglio (Wayne State University, Detroit, MI) (14). Both H5V and CD3-21 cells were grown in DMEM, 10% FBS, and 5 μ g/ml gentamicin.

In Vivo Matrigel Assay. This assay was performed as described (15). Briefly, Matrigel (liquid at 4°C) was mixed with 150 ng/ml bFGF alone or in combination with IP-10, TGF- β , IL-8, Mig, RANTES, or MCAF, each at a final concentration of 400 ng/ml. Matrigel alone or with bFGF, or with bFGF plus the test cytokine (total vol 0.5 ml), was injected subcutaneously into the midabdominal region of the BALB/c nude mouse. After injection, the Matrigel polymerized to form a plug. After 7 d, the animals were killed, and the Matrigel plugs were removed together with the abstract epidermis and dermis, fixed in 10% neutral buffered formalin solution (Sigma Chemical Co.), and embedded in paraffin. Histological sections were stained with Masson's trichrome. The vessel area in the histological sections was measured using a computerized digital analyzer (Optomax, Hollis, NH) as described (15). The mean area per field ($\times 10^3 \mu\text{m}^2$) from 15 fields (20 \times) was calculated.

Cell Proliferation. FBHE, HUVEC, H5V, and CD3-21 cells were used routinely before the 15th passage. After trypsinization, the cells were plated in triplicate cultures of 1×10^3 or 8×10^3 cells in 0.2 ml complete medium with or without additives in a 96-well flat-bottom plate. The plates were incubated for 1–7 d. DNA synthesis was determined by [^3H]thymidine deoxyribose uptake (0.5 $\mu\text{Ci}/\text{well}$, 6.7 Ci/mmol; New England Nuclear, Boston, MA)

during the last 6 or 18 h of culture. Cells were detached by freezing and thawing.

Cell Attachment Assay. This assay was performed as described (16). HUVEC cells were plated (in four replicates, 4×10^4 cells/well in complete medium) on BSA-precoated 48-well plates, and IP-10 was added at final concentrations of 0, 15, 45, 135, 400, or 1,200 ng/ml. After incubation for 1 h, the supernatant was aspirated, and the cells were fixed and stained using Diff-Quick (Baxter Scientific Products, McGaw Park, IL).

Cell Migration Assay. Cell migration was measured as described (17). Two 2-mm scratches were made in each well of a 6-well plate containing confluent HUVEC using a modified rubber cell scraper. The wells were rinsed, and 1.5 ml complete medium containing 0, 15, 45, 135, 400, or 1,200 ng/ml IP-10 was added. After 24 h of incubation, two additional scratches per well were made as reference marks, the medium was aspirated, and the cells were fixed and stained using Diff-Quick. Cell migration into the scratched area was evaluated under an inverted phase microscope.

Endothelial Tube Formation on Matrigel. Tube formation was evaluated as described (18). Matrigel (320 $\mu\text{l}/\text{well}$), used at 4°C to coat a 24-well plate, was allowed to polymerize at 37°C for 2 h. HUVEC, $4\text{--}6 \times 10^4$ cells/well, in a final vol of 1 ml HUVEC complete medium containing 0, 15, 45, 135, 400, or 1,200 ng/ml IP-10, were plated on the Matrigel substratum. After an 18-h incubation, the medium was aspirated, and the adherent cells were fixed and stained using Diff-Quick. Tube formation was examined visually, and total tube area per well was quantitated by the digital analyzer. The assay was performed in duplicate.

Statistical Analysis. Arithmetic means, standard deviations, and Student's *t* tests were calculated by conventional formulas using Systat for the Macintosh (Systat Inc., Evanston, IL).

Results

IP-10 Inhibits Neovascularization In Vivo To assess the effects of IP-10 on neovascularization in vivo, we have used a mouse model in which subcutaneous injection of Matrigel impregnated with bFGF rapidly induces new vessel formation (15). The results of a representative experiment in which groups of athymic mice were injected either with Matrigel alone (0.5 ml) (Fig. 1 A); Matrigel (0.5 ml) plus bFGF (150 ng/ml) (Fig. 1 B), or Matrigel (0.5 ml) plus bFGF (150 ng/ml) plus IP-10 (400 ng/ml) (Fig. 1 C) are depicted in Fig. 1. Microscopic examination of the Matrigel plugs removed from the mice 7 d after injection revealed the presence of only a few endothelial cells invading the Matrigel alone plug (A). In contrast, abundant endothelial cells, often organized to form blood vessels containing red blood cells, were present in the Matrigel plus bFGF plug (B). Addition of IP-10 to Matrigel plus bFGF resulted in marked reduction in the number of endothelial cells invading the plug and the absence of blood vessels (C). Quantitative analysis of this experiment, which included five nude mice per treatment group, is shown in Fig. 1 D. Plugs of Matrigel alone contained very few endothelial cells (mean surface area $483.8 \times 10^3 \mu\text{m}^2$). Matrigel plugs impregnated with bFGF contained ~ 13 -fold more cells compared with plugs of Matrigel alone (mean surface area $6,511.2 \times 10^3 \mu\text{m}^2$). Matrigel plugs with IP-10 added together with bFGF contained significantly fewer cells compared to Matrigel plus bFGF plugs (mean surface area

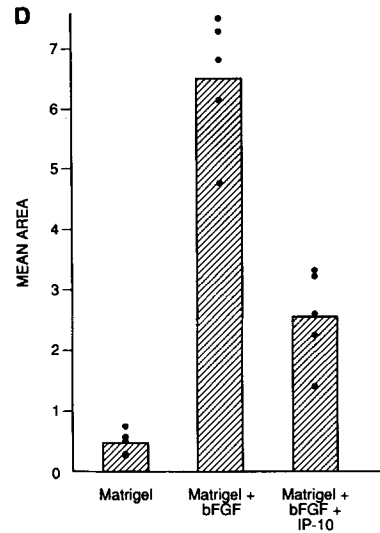
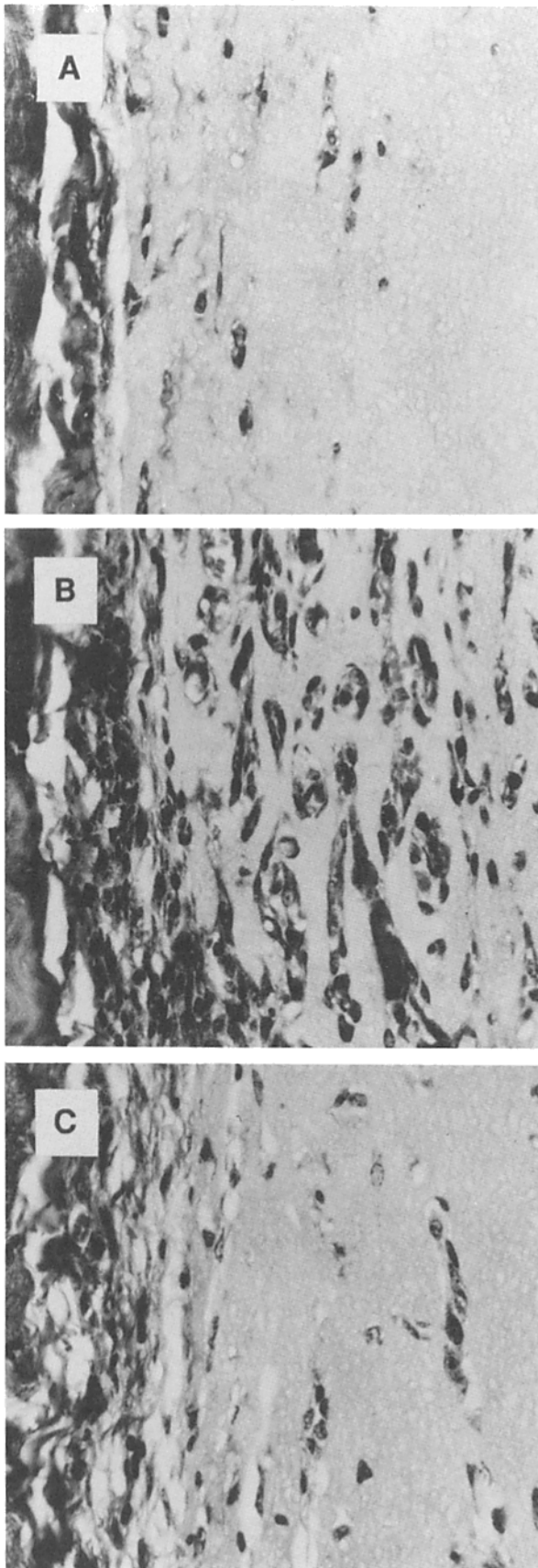


Figure 1. Effect of IP-10 on neovascularization in vivo. Groups of five female BALB/c nu/nu mice were injected subcutaneously with Matrigel alone, Matrigel plus bFGF, or Matrigel plus bFGF and IP-10. Plugs were removed 7 d after injection, and histologic sections were stained with Masson's trichrome. (A) Histology ($\times 400$) of a representative Matrigel alone plug, (B) Matrigel plus bFGF plug, and (C) Matrigel plus bFGF and IP-10 plug. In A, B, and C, the left margin coincides with the edge of the plug. (D) Quantification of angiogenesis. Results are expressed as mean Matrigel surface area ($\times 10^3 \mu\text{m}^2$) occupied by cells, as determined by a semiautomated digitalized analyzer. Each dot represents the mean surface area ($\times 10^3 \mu\text{m}^2$) for each mouse and reflects 15 readings on nonoverlapping Matrigel fields.

$2,568.0 \times 10^3 \mu\text{m}^2$, $P = 0.0002$). These experiments demonstrated that IP-10 can act as a potent inhibitor of bFGF-induced neovascularization in vivo.

We wished to confirm these observations and to test whether other cytokines/chemokines might also act as inhibitors of neovascularization measured by this in vivo assay system. As shown in Table 1, IP-10 consistently inhibited neovascularization of Matrigel plugs containing bFGF. When added to Matrigel alone, without bFGF, IP-10 had at most a slight inhibitory effect. Mig and IL-8, two additional members of the α chemokine family, had little or no inhibitory effect on angiogenesis induced by bFGF and Matrigel. RANTES and MCAF, members of the β chemokine family, had no inhibitory effect on neovascularization induced by bFGF and Matrigel. As reported (15), TGF- β had little or no effect on neovascularization in this assay system. These experiments demonstrated that IP-10 can be a potent inhibitor of neovascularization induced by bFGF in vivo, and they suggest that inhibition of angiogenesis is not common to all chemokines.

Effects of IP-10 on Endothelial Cell Proliferation, Attachment, Migration and Differentiation. Angiogenesis is known to be a complex process that requires endothelial cell proliferation, migration, and differentiation into tubelike structures (1). Since IP-10 inhibited angiogenesis in vivo, we wished to dissect its mode of action. First, we tested whether IP-10 can inhibit endothelial cell proliferation. HUVEC and FBHE are growth

Table 1. Effects of Cytokines and Chemokines on Angiogenesis In Vivo

Additions to Matrigel	Mean surface area	± SD
	× 10 ³ μm ²	
None	554	196
bFGF	5,511	1,404
bFGF + IP-10	1,317	389
IP-10	355	128
None	484	209
bFGF	6,511	1,097
bFGF + IP-10	2,568	769
bFGF + TGF-β	9,131	2,113
None	997	34
bFGF	7,060	820
bFGF + IP-10	2,118	447
bFGF + IL-8	5,662	867
bFGF + Mig	6,303	824
None	665	152
bFGF	6,140	854
bFGF + IP-10	1,826	858
bFGF + RANTES	5,856	657
bFGF + MCAF	6,196	820

BALB/c nu/nu female mice (five mice per condition in each experiment) were injected subcutaneously with either Matrigel alone (0.5 ml), Matrigel plus bFGF (150 ng/ml), Matrigel plus IP-10 (400 ng/ml), or Matrigel plus bFGF (150 ng/ml) plus one of the indicated cytokine/chemokines (all at 400 ng/ml). The Matrigel plugs were removed after 7 d and processed for histology. The results reflect the mean Matrigel surface area occupied by cells (± SD) for each group of mice.

factor-dependent primary endothelial cell cultures found to grow optimally in vitro with ECGS and bFGF, respectively. The murine heart endothelioma cell line H5V and the murine pulmonary microvascular endothelial cell line CD3-21 are immortalized, growth factor-independent endothelial cell lines. IP-10 (400 ng/ml) had little or no effect on growth factor (bFGF or ECGS)-induced or spontaneous endothelial cell proliferation when measured after 7 d of culture (Table 2). Also, IP-10 had little or no effect on growth factor-induced HUVEC proliferation when measured after 30 h of culture, even at 2 μg/ml and in the absence of heparin. As expected, TGF-β inhibited bFGF-induced proliferation of FBHE cells, and PF-4, in the absence of heparin, inhibited the proliferation of HUVEC at 40 μg/ml but not at 2 μg/ml. Time course experiments (not shown) confirmed that IP-10 failed to inhibit endothelial cell proliferation at 1, 3, and 5 d. Dose response experiments (not shown) demonstrated that IP-10 failed to inhibit endothelial cell growth at doses ranging

between 10 ng/ml and 2 μg/ml. Thus, IP-10 is not an inhibitor of endothelial cell growth in vitro, suggesting that inhibition of cell division is not the mechanism by which IP-10 inhibits angiogenesis in vivo.

To assess whether IP-10 interferes with the migration and attachment of endothelial cells, HUVEC cells were tested for their ability to attach to BSA-coated plastic wells (16) and to migrate (17), in the presence or absence of IP-10 (15–1,200 ng/ml). As measured in these assays, neither endothelial cell attachment nor migration was affected by IP-10 at all tested doses (results not shown).

We next tested whether IP-10 inhibits the differentiation of endothelial cells into tubelike structures, an essential step to new blood vessel formation (16). As reported (16), when endothelial cells (HUVEC) were cultured for 12–18 h on a Matrigel substrate, they rapidly aligned with one another and formed an intricate network of tubelike structures (Fig. 2 A). In the presence of IP-10, the endothelial cells formed small aggregate structures, and the network of tubelike structures was strikingly less extensive than in control cultures (Fig. 2 B). This effect of IP-10 was not due to inhibition of endothelial cell attachment to the Matrigel monolayer or to inhibition of cell survival and proliferation during incubation, because, at the end of culture, with or without IP-10, both similar numbers of nonadherent cells were recovered (~10% of the initial input), and similar numbers of nuclei were counted on the Matrigel monolayer. Measurement of the total tube area by a semiautomated digital analyzer confirmed the visual observation that IP-10 inhibits endothelial cell differentiation into tubelike structures (Fig. 3 C). This effect was dose dependent, and the surface area reduction was significant at all IP-10 doses tested ($P \leq 0.001$). These experiments demonstrated that IP-10 inhibits endothelial cell differentiation into capillarylike structures in vitro and suggested a mechanism whereby IP-10 could inhibit angiogenesis in vivo.

Discussion

We have shown that human IP-10 is a potent inhibitor of angiogenesis in vivo. When bFGF, an angiogenic factor, was added to Matrigel and injected subcutaneously into mice, a vigorous local angiogenic response was induced. However, when IP-10 was added to bFGF-containing Matrigel, the angiogenic response was markedly reduced. In vitro testing showed that IP-10 does not inhibit endothelial cell proliferation occurring either spontaneously or after induction by bFGF or ECGS, nor does it inhibit endothelial cell attachment and migration. However, IP-10 dose dependently inhibited endothelial cell differentiation into branching networks of tubular structures in vitro, a complex process requiring interaction of endothelial cells with components of the extracellular matrix (16). These findings demonstrated that IP-10 is an inhibitor of angiogenesis and suggested that IP-10 suppresses endothelial cell differentiation into capillary structures.

IP-10, a member of the α chemokine family, was initially described as an immediate early gene induced by IFN-γ in the histiocytic lymphoma cell line U937 (19). Activated human mononuclear cells, keratinocytes, fibroblasts, endothelial cells,

Table 2. *Effects of IP-10 on Endothelial Cell Proliferation In Vitro*

Additions to culture	Proliferation*	
	Experiment 1	Experiment 2
	<i>cpm/culture</i>	
FBHE†		
Medium	1,094	1,294
bFGF	8,667	10,664
bFGF + IP-10 (400 ng/ml)	8,298	9,421
bFGF + TGF- β (10 ng/ml)	193	315
H5V‡		
Medium	71,753	116,102
Medium + IP-10 (400 ng/ml)	70,148	108,276
Medium + TGF- β (10 ng/ml)	n.d.	72,548
CD3-21‡		
Medium	755	695
Medium + IP-10 (400 ng/ml)	833	754
Medium + TGF- β (10 ng/ml)	724	n.d.
HUVEC‡		
Medium	253	191
ECGS	8,648	7,965
ECGS + IP-10 (400 ng/ml)	6,315	8,050
ECGS + TGF- β (10 ng/ml)	6,441	7,138
HUVEC§		
Medium	254	395
ECGS	1082	915
ECGS + IP-10 (2 μ g/ml)	1092	1784
ECGS + PF4 (2 μ g/ml)	979	674
ECGS + PF4 (40 μ g/ml)	406	546

* [³H]Thymidine was added during the final 18 h of culture. The results are expressed as mean cpm of triplicate cultures (SDs within 12% of the mean). *n.d.*, not done.

† The endothelial cells/cell lines FBHE, HUVEC, H5V, and CD3-21 (1×10^3 cells/0.2 ml well) were cultured for 7 d with or without IP-10 or TGF- β .

§ HUVEC (8×10^3 cells/0.2 ml well) were cultured without heparin for 30 h with or without IP-10 or PF-4.

and T cells also express the IP-10 gene (19). Both the human IP-10 gene and the presumed murine homologue, *crg-2*, code for a secreted mature protein with a predicted molecular mass of \sim 8.6 and 8.7 kD, respectively (20, 21). Originally thought to be involved in inflammatory processes because of its inducibility by IFN- γ and structural similarity to PF-4 and β -thromboglobulin, IP-10 appears to be multifunctional. It inhibited *in vitro* colony formation by human bone marrow hematopoietic cells (22) and exerted a potent antitumor effect *in vivo* (23). Recently, IP-10 was reported to be a chemoattractant for human monocytes and activated T lymphocytes

and to promote T cell adhesion to endothelial cells (24). However, IP-10 has not been found previously to act as an inhibitor of angiogenesis.

It is of interest that IP-10 inhibited angiogenesis *in vivo* and endothelial cell differentiation *in vitro* without inhibiting endothelial cell proliferation *in vitro*. Fumagillin, a product of *Aspergillus fumigatus fresenious* (10); AGM-1470, a synthetic homologue of fumagillin (10); thrombospondin (4), a matrix glycoprotein secreted by a variety of cell types; and the recently identified angiostatin, a fragment of plasminogen (25), have all been shown to potently inhibit endothelial cell

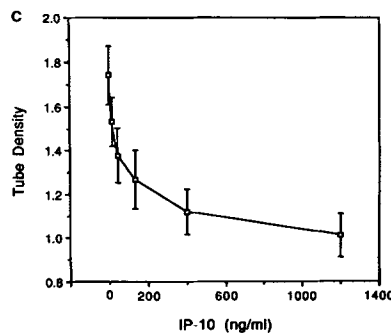
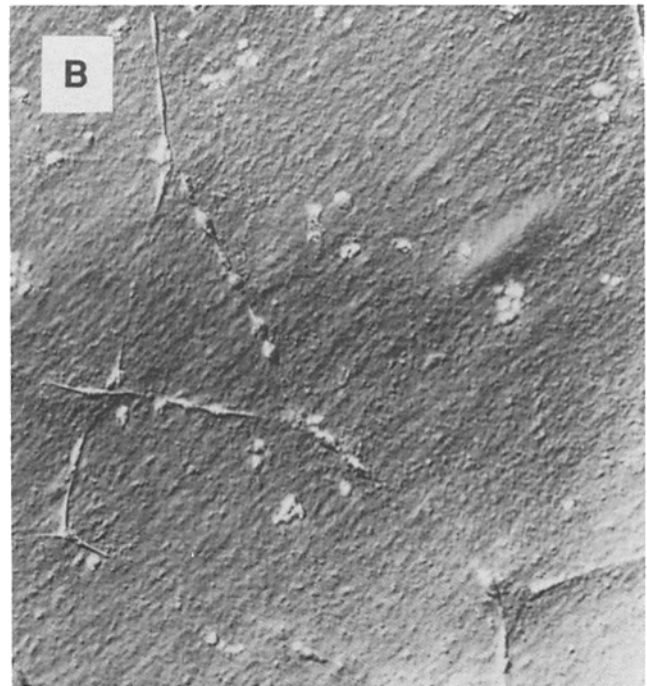
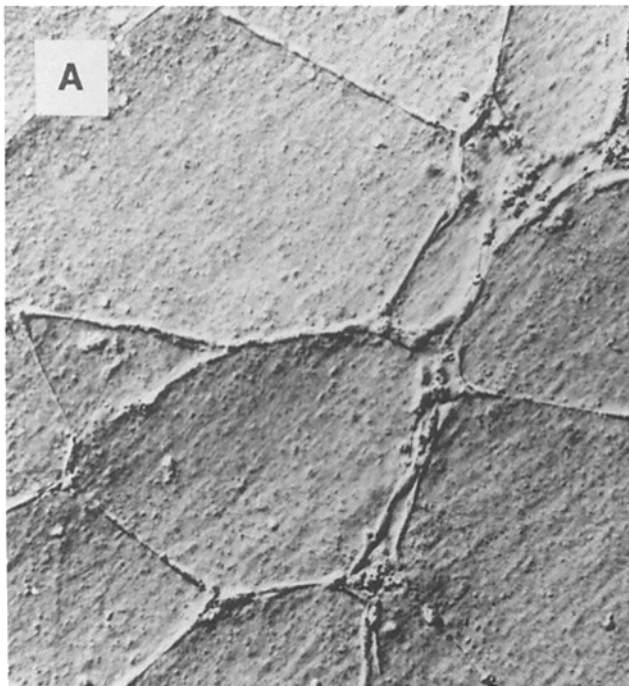


Figure 2. Effect of IP-10 on endothelial cell differentiation. HUVEC cells (6×10^4) were plated on Matrigel-coated 24-well plates in either complete medium alone or in complete medium supplemented with IP-10 (15–1,200 ng/ml). After an 18-h incubation, the cells were stained with Diff-Quick. (A) Microscopic morphology ($\times 100$) of HUVEC cultured in medium alone, and (B) in medium supplemented with 1,200 ng/ml IP-10. (C) Measurement of the area occupied by tubelike structure using a semiautomated digitalized analyzer. The results reflect the mean (\pm SD) of 10 separate experiments.

proliferation in vitro and to suppress angiogenesis in vivo. But TGF- β , a potent inhibitor of endothelial cell proliferation in vitro, does not inhibit, and perhaps stimulates, angiogenesis in vivo (3, 15). IFN- α , the first angiogenesis inhibitor to reach clinical trials, has produced beneficial results in the treatment of certain hemangioendotheliomas (26), but the mechanisms by which it acts are poorly understood. A multistep and complex process such as angiogenesis is likely to be under multiple regulatory controls. Inhibition of angiogenesis in vivo by a drug may or may not be the result of a direct inhibition of endothelial cell growth.

One of the first angiogenesis inhibitors to be identified was PF-4 (8), which, like IP-10, is a member of the α chemokine family. In vitro, PF-4 is a potent inhibitor of growth factor-dependent endothelial cell proliferation (HUVEC), albeit at high ($\sim 25 \mu\text{g/ml}$) concentrations. Another member of the α chemokine family, IL-8, was reported to stimulate umbilical vein endothelial cell proliferation in vitro and to be potentially angiogenic in vivo (27, 28). The observations that IL-8 acts as an angiogenic factor and that PF-4 and IP-10 act as inhibitors of angiogenesis suggest that, among chemokines, stimulators and inhibitors of angiogenesis might seg-

regate with the presence or absence of the Glu-Leu-Arg motif. IL-8 is representative of the subset of α chemokines that contain a Glu-Leu-Arg motif near their NH₂ termini (29), a motif absent in both PF-4 and IP-10 (29). It is of interest that we found human Mig not to be an inhibitor of angiogenesis, because human Mig also lacks the Glu-Leu-Arg motif (30). Thus, inhibition of angiogenesis in the Matrigel assay is not a property shared among members of any of the recognized subsets of the chemokine superfamily.

Tumor cells are dependent upon new vessel formation to grow locally and metastasize (1, 2). In some cases, tumor cells themselves are potently angiogenic, partly because of their failure to secrete factors that inhibit angiogenesis while continuing the secretion of factors that stimulate angiogenesis (25). The observation that genetically engineered tumor cells that produce high levels of IP-10 lost their tumorigenicity (23) raises the possibility that this phenotypic switch might be attributable, in part, to the antiangiogenic effect of IP-10. The newly described biological property of IP-10 to inhibit angiogenesis suggests the possibility that this chemokine might be considered as a potential anticancer therapeutic for future clinical development.

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