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Scatter factor induces blood vessel formation *in vivo*

(hepatocyte growth factor/angiogenesis/endothelium/psoriasis/plasminogen activator)

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ABSTRACT **Scatter factor (also known as hepatocyte growth factor) is a glycoprotein secreted by stromal cells that stimulates cell motility and proliferation. *In vitro*, scatter factor stimulates vascular endothelial cell migration, proliferation, and organization into capillary-like tubes. Using two different *in vivo* assays, we showed that physiologic quantities of purified native mouse scatter factor and recombinant human hepatocyte growth factor induce angiogenesis (the formation of new blood vessels). The angiogenic activity was blocked by specific anti-scatter factor antibodies. Scatter factor induced cultured microvascular endothelial cells to accumulate and secrete significantly increased quantities of urokinase, an enzyme associated with development of an invasive endothelial phenotype during angiogenesis. We further showed that immunoreactive scatter factor is present surrounding sites of blood vessel formation in psoriatic skin. These findings suggest that scatter factor may act as a paracrine mediator in pathologic angiogenesis associated with human inflammatory disease.**

Scatter factor (SF) was described as a cytokine secreted by fibroblasts (1, 2) and vascular smooth muscle cells (3) that disperses cohesive epithelial colonies and stimulates cell motility. SF is identical to hepatocyte growth factor (HGF) (4, 5), an independently characterized serum mitogen (6, 7). SF induces kidney epithelial cells in a collagen matrix to form branching networks of tubules, suggesting that it can also act as a morphogen (8). SF (HGF) is a basic heparin-binding glycoprotein consisting of a heavy (58 kDa) and a light (31 kDa) subunit (6, 7, 9–12). It has 38% amino acid sequence identity with the proenzyme plasminogen (7) and is thus related to the blood coagulation family of proteases. Its receptor in epithelium was identified as the c-*met* protooncogene product, a transmembrane tyrosine kinase (13, 14).

Angiogenesis is a multistep process in which endothelial cells focally degrade and invade through their own basement membrane, migrate through interstitial stroma toward an angiogenic stimulus, proliferate proximal to the migrating tip, organize into blood vessels, and reattach to newly synthesized basement membrane (15). These processes are controlled by soluble factors and by the extracellular matrix (15, 16). *In vitro*, SF stimulates endothelial chemotactic and random migration in Boyden chambers (11), migration from carrier beads to flat surfaces (11, 17), formation of capillary-like tubes (18), and DNA synthesis (9). Preliminary studies suggest SF may also induce endothelial secretion of plasminogen activators (PAs) (18). Proteases such as PAs are required during the early stages of angiogenesis, in which endothelial cells degrade extracellular matrix. Since endothelial cell migration, proliferation, and capillary tube formation occur during angiogenesis, we suspected that SF might exhibit angiogenic activity *in vivo*.

MATERIALS AND METHODS

SF Preparations. Mouse SF was purified from serum-free culture medium from *ras*-transformed NIH/2 3T3 cells (clone D4) by cation-exchange chromatography (11) followed by immunoaffinity chromatography and ultrafiltration (19). Recombinant human HGF (rhHGF) (7) was provided by Toshikazu Nakamura (Kyushu University, Fukuoka, Japan).

Antibody Preparations. Antisera to native human placental SF and rhHGF were prepared by immunizing rabbits with purified factors (5, 19). A chicken egg yolk antibody to human placental SF was prepared by immunizing two White Leghorn hens, 22–24 weeks old, with 500 μg of human placental SF emulsified in complete Freund's adjuvant (20). Booster injections were given 14 and 28 days later, and the eggs were collected daily. The IgG fraction from seven eggs was extracted and partially purified (21). The final preparation contained 80 μg of protein per ml in phosphate-buffered saline (PBS). Antibody specificity was established by recognition of mouse and human SFs on immunoblots, specific binding of SF to antibody-Sepharose columns, and inhibition of the *in vitro* biologic activities of mouse and human SFs (5, 19).

PA Assays. Bovine brain microvessel endothelial cells (BBEC) were isolated from brain cortex after removal of the pia mater, identified as endothelial, and cultured by standard techniques (17). BBEC (passage 10–12) at about 80% confluency in 60-mm Petri dishes were treated with mouse SF for 24 hr, washed, and incubated for 6 hr in 2.5 ml of serum-free Dulbecco's modified Eagle's medium (DMEM) to collect secreted proteins. The cells were washed, scraped into PBS, collected in 0.5 ml of PBS by centrifugation, and lysed by sonication. Aliquots of medium and cell lysates were assayed for PA activity by a two-step chromogenic reaction (22). Human high molecular weight urokinase (American Diagnostica, Greenwich, CT) was used as the standard. The protein content of the lysate was determined by using the Bradford dye-binding assay (Bio-Rad).

Murine Angiogenesis Assay. Angiogenesis was assayed as growth of blood vessels from subcutaneous tissue into a solid gel of basement membrane containing the test sample (23). Matrigel (7 mg in 0.5 ml; Collaborative Research) in liquid form at 4°C was mixed with SF and injected into the abdom-

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Abbreviations: SF, scatter factor; HGF, hepatocyte growth factor; rhHGF, recombinant human HGF; BBEC, bovine brain endothelial cells; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; PA, plasminogen activator; TGFβ, transforming growth factor β; TNF, tumor necrosis factor; IL-6, interleukin 6.

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inal subcutaneous tissues of athymic XID *nude beige* mice or C57BL/6 mice. Matrigel rapidly forms a solid gel at body temperature, trapping the factor to allow slow release and prolonged exposure to surrounding tissues (24). After 10 days, the mice were sacrificed and the Matrigel plugs were excised and fixed in 4% formaldehyde in phosphate buffer. Plugs were embedded in paraffin, sectioned, stained with Masson's trichrome (which stains endothelial cells reddish-purple and stains the Matrigel violet or pale green), and examined for ingrowth of blood vessels. Vessel formation was quantitated from stained sections using the Optimax digital image analyzer connected to an Olympus microscope (25). Prior studies showed an excellent correlation of the stained area and factor VIII staining (23). Results were expressed as mean vessel area per field ± SEM (arbitrary units) or as total vessel area (mm^2) in 20 random fields.

Rat Cornea Angiogenesis Assay. Angiogenesis was assayed in the avascular rat cornea, as described before (26). Briefly, test samples were combined 1:1 with a sterile solution of Hydron (Interferon Laboratories, New Brunswick, NJ) and air-dried overnight. A 5-μl pellet was inserted into a surgically created pocket in the corneal stroma and positioned 1–1.5 mm from the limbus. Corneas were examined daily with a dissecting microscope for up to 7 days for capillary growth. Assay responses were scored as positive if sustained directional ingrowth of capillary sprouts and hairpin loops occurred during the observation period. Responses were scored as negative either when no neovascularization was detected or when only an occasional sprout or hairpin loop was observed that showed no evidence of sustained directional ingrowth. After 7 days, corneas were perfused with colloidal carbon, and whole-mount preparations were examined and photographed.

Immunohistochemistry. Five-micrometer-thick cryostat sections were prepared from biopsy samples of plaques or of areas of normal skin in patients with active psoriasis. The sections were stained by using an avidin–biotin immunoperoxidase technique (27). The chromogen was Texas red conjugated to avidin. The primary antibody was rabbit polyclonal antiserum to purified native human placental SF or to rhHGF (1:1000 dilution). Nonimmune rabbit serum (1:1000) was used as a negative control.

RESULTS

We used two different *in vivo* assays to evaluate the angiogenic activity of mouse SF. In the first assay, the murine angiogenesis assay (23), samples mixed with Matrigel, a matrix of reconstituted basement membrane (24), were injected subcutaneously into mice. After 10 days, the mice were sacrificed for histologic and morphometric analysis of Matrigel plugs. Grossly, control plugs were pale pink, while plugs containing SF were bright red and often contained superficial blood vessels (Fig. 1 *A* and *B*). Histologic analysis showed little cellularity in control plugs (Fig. 2*a*). Plugs containing 2 ng of SF often had increased numbers of cells (Fig. 2*b*), 90% of which stained for factor VIII antigen, an endothelial cell marker (not shown). At 20 ng of SF, cell number was increased, and vessels were present (Fig. 2*c*). At 200 ng of SF, plugs were even more cellular, with endothelial cells making up 50–60% of the cell population. Many large vessels containing smooth muscle cells were seen (Fig. 2*d*). Morphometric analysis of vessel area (25) revealed a dose-dependent angiogenic response in athymic (Fig. 1*C*) and C57BL (Fig. 1*D*) mice, with half-maximal and maximal responses at about 20 and 200 ng, respectively. Histologic examination at day 10 showed no evidence of inflammation in SF-containing plugs in athymic mice. In C57BL, no inflammation was observed at ≤200 ng of SF, but leukocytic

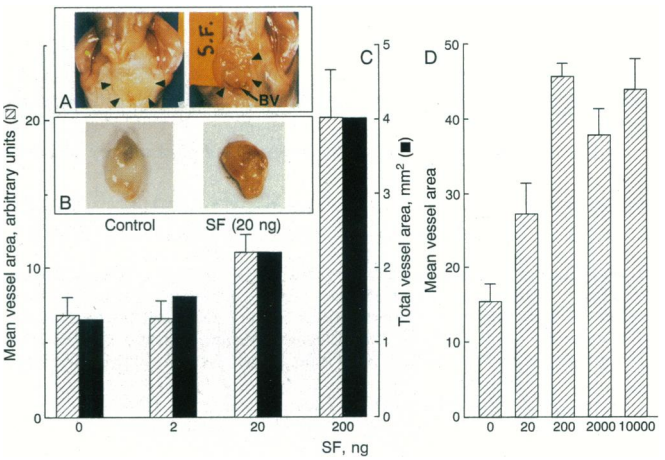

FIG. 1. Murine angiogenesis assay. Matrigel mixed with purified mouse SF was injected subcutaneously into mice (four mice per SF dose). After 10 days, mice were sacrificed and Matrigel plugs were excised and fixed. The photographs show plugs (arrowheads) before (*A*) and after (*B*) excision. The arrow points to a superficial blood vessel (BV) in an SF-containing plug. Plugs were embedded in paraffin, sectioned, and stained with Masson's trichrome. Vessels were quantitated by digital image analysis (25) for athymic mice (*C*) and for C57BL mice (*D*).

infiltration was present in tissue surrounding the plugs at ≥2000 ng of SF.

In the second assay, samples were implanted in the avascular rat cornea to allow ingrowth of blood vessels from the limbus (26). Control implants gave no positive responses (Table 1, Fig. 3*A*), while implants containing mouse SF induced a dose-dependent corneal neovascularization. Responses at 50 ng (Fig. 3*B*) were reduced in intensity compared with those at 100 and 500 ng (Fig. 3 *C* and *D*, respectively). The maximal response to SF was observed at doses of ≥100 ng and was similar to the response to 150 ng of human basic FGF, a positive control (Fig. 3*E*). rhHGF also induced angiogenesis in the rat cornea (Table 2). At 100 ng, positive responses were observed in four of five implants; at 500 ng of rhHGF, all five implants gave positive responses. Chicken and rabbit antibodies to human placental SF strongly inhibited the angiogenic responses to mouse SF and rhHGF but not to basic FGF (Table 2). To assess inflammation, corneas were examined by direct stereomicroscopy daily for the duration of the experiments. Corneas chosen at random were examined histologically at 6, 12, and 24 hr and at 3, 5, and 7 days after implantation of SF and control pellets. Inflammation was not detected at lower angiogenic doses of SF (50–500 ng of mouse SF, 100 ng of rhHGF). At higher doses (1000 ng of mouse SF, 500 ng of rhHGF), a prominent inflammatory infiltrate was often observed. The majority of cells were monocytes and macrophages, as judged by morphology and immunostaining for F4/80, a macrophage/monocyte marker.

PAs convert plasminogen into plasmin, a potent serine protease that lyses fibrin clots, degrades components of extracellular matrix, and activates enzymes (e.g., procollagenases) that further degrade matrix (28). SF induced large dose-dependent increases in secreted (Fig. 4*A*) and cell-associated (Fig. 4*B*) PA activity in microvascular endothelium (BBEC). Total PA activity (secreted plus cell-associated) was increased 4-fold relative to control when SF was present at 20 ng/ml (≈0.2 nM). Similar results were obtained in large vessel endothelium (not shown). Most of the secreted and cell-associated PA activity in BBEC was blocked by antibodies to urokinase but not by antibodies to tissue PA (Fig. 4 *D* and *E*).

Angiogenesis is often associated with chronic inflammatory diseases. Psoriasis is a common inflammatory skin

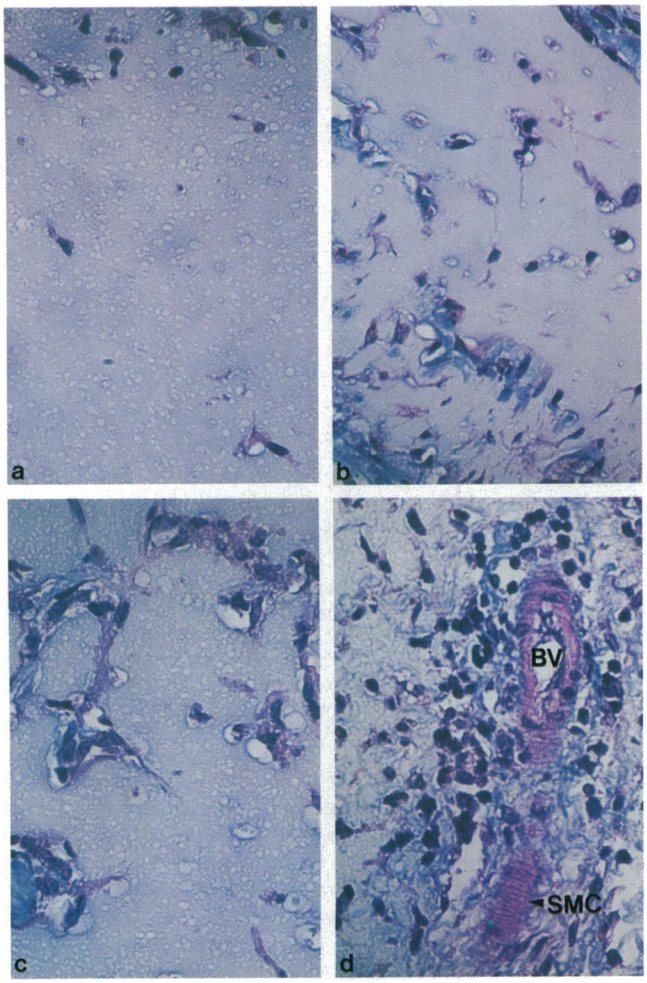

FIG. 2. Microscopic appearance of Matrigel plugs. Sections of plugs from athymic mice which contained 0 (control *a*), 2 (*b*), 20 (*c*), and 200 (*d*) ng of SF, respectively. (×200.) BV, blood vessel; SMC, smooth muscle cell.

disease characterized by prominent epidermal hyperplasia and neovascularization in the dermal papillae. Frozen sections of biopsy samples from psoriatic plaques from 10 patients each showed positive immunohistochemical staining

Table 1. Neovascular responses induced in rat corneas by SF

Content of pellet	Corneal neovascularization: Positive responses	%
Negative controls		
Sham implant	0/3	0
Hydron	0/2	0
PBS	0/2	0
Positive control		
Basic FGF (150 ng)	4/4	100
SF		
5 ng	0/4	0
50 ng	3/5*	60
100 ng	5/5	100
500 ng	5/5	100
1000 ng	5/5†	100

The assay and criteria for a positive response are described in *Rat Cornea Angiogenesis Assay*. FGF, fibroblast growth factor.
*Responses were much weaker in intensity compared with implants containing 100 or 500 ng of SF.
†Corneas showed significant inflammation.

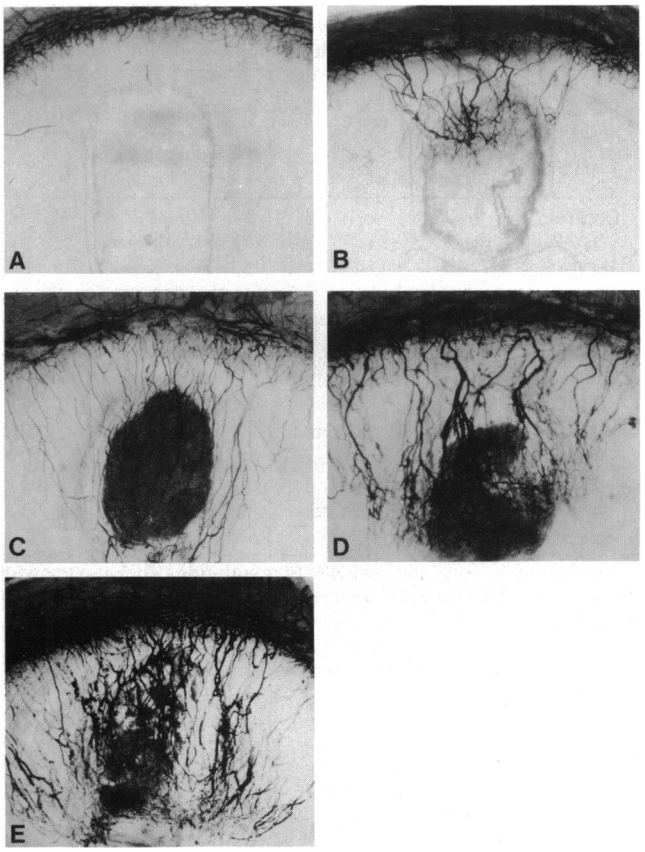

FIG. 3. SF-induced angiogenesis in rat corneas. Whole-mount preparations of colloidal carbon-perfused corneas 7 days after implantation of Hydron pellets. (×14.) The darkened area in the center of the perfused cornea is the reflection of the implant. No angiogenic response was observed in control pellets containing PBS (*A*). At 50 ng of SF (*B*), the response was positive but weak in comparison with higher concentrations of SF. At 100 (*C*) and 500 (*D*) ng of SF, strong positive responses were seen, with capillary sprouts and hairpin loops surrounding the implants. A strong angiogenic response was induced by 150 ng of basic FGF, a positive control (*E*).

for SF in spindle-shaped and mononuclear cells within the dermal papillae and papillary dermis. Antisera to human placental SF and rhHGF gave an identical staining pattern (illustrated in Fig. 5*A*). SF-positive cells were arranged in a perivascular distribution; cells of the blood vessel wall did not stain for SF (Fig. 5*C*). Normal skin from psoriasis patients or from normal subjects showed little or no staining for SF (Fig. 5*D*). Sections from psoriatic plaques treated with nonimmune serum as the primary antibody (negative control) showed no staining (Fig. 5*B*).

DISCUSSION

Physiologic quantities of SF [100–200 ng (≈1–2 pmol)] induced strong angiogenic responses in two *in vivo* assays. It is likely that this angiogenic activity is due, in part, to direct effects on endothelium since: (*i*) SF stimulates endothelial migration, proliferation, and tube formation *in vitro*; (*ii*) histologic studies showed no evidence of inflammation at SF doses that gave strong angiogenic responses; and (*iii*) anti-SF antibodies blocked the angiogenic responses. We also found that SF stimulates endothelial cell expression of urokinase. Urokinase bound to its specific cell surface receptor is thought to mediate focal, directed, extracellular proteolysis, which is required for endothelial cell invasion and migration during the early stages of angiogenesis (28).

Table 2. Neovascular responses induced in rat corneas by native mouse SF and rhHGF with or without antibody (Ab)

Content of pellet	Corneal neovascularization: Positive responses	%
Controls		
Hydron + PBS	0/8	0
Chicken Ab	0/4	0
Rabbit Ab (Ab 978)	0/3	0
Basic FGF (150 ng)	3/3	100
Basic FGF (150 ng) + rabbit Ab	3/3	100
Factor ± Ab		
Mouse SF (100 ng)	3/3	100
Mouse SF (100 ng) + chicken Ab	1/5*	20
rhHGF (100 ng)	4/5	80
rhHGF (500 ng)	5/5†	100
rhHGF (100 ng) + chicken Ab	2/5*	33
rhHGF (100 ng) + rabbit Ab	0/5	0

Antibodies were diluted in PBS. Final dilutions after mixing with Hydron were 1:20 for the chicken antibodies and 1:200 for the rabbit antibodies.
*Responses scored as positive were very weak.
†This concentration of rhHGF was inflammatory.

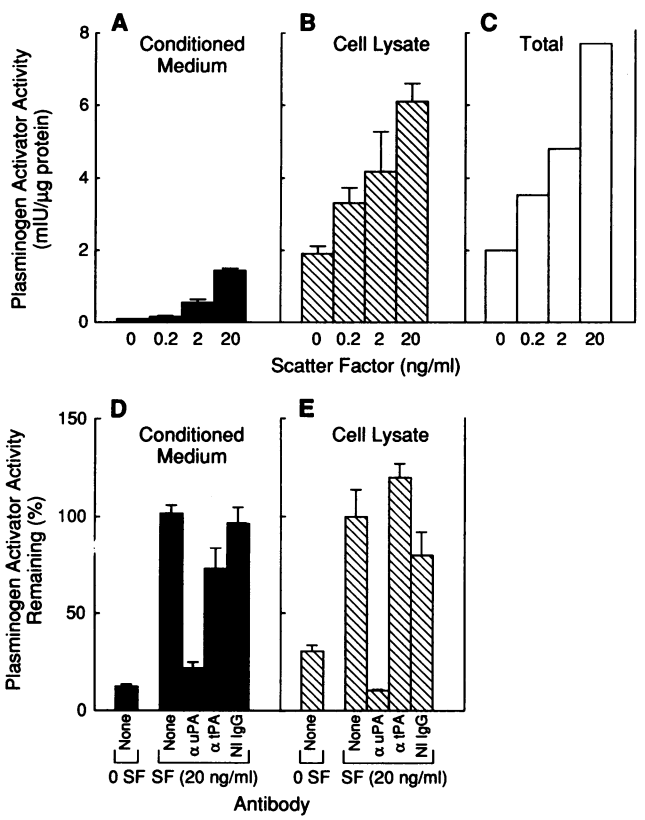

FIG. 4. Stimulation of PA expression by SF. BBEC treated with mouse SF for 24 hr were assayed for secreted and intracellular PA activity. (*A–C*) Secreted activity during a 6-hr collection interval (*A*), intracellular activity (*B*), and total (secreted plus intracellular) activity (*C*). IU, international unit. (*D* and *E*) Medium (*D*) and lysates (*E*) from cells treated with SF at 20 ng/ml were assayed in the presence of goat anti-human urokinase IgG (αuPA), goat anti-human tissue type PA IgG (αtPA), or goat nonimmune IgG (NI IgG) (200 μg/ml) (American Diagnostica, Greenwich, CT) to determine the type of PA present. Values represent mean ± SEM of triplicate determinations.

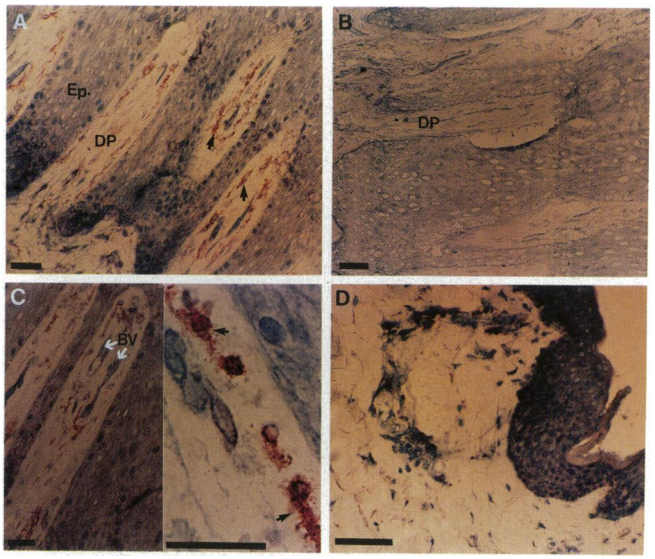

FIG. 5. Immunohistochemical staining of skin biopsy samples for SF. (*A–C*) Psoriatic plaques. (*D*) Normal skin from a patient with psoriasis. The primary antibody was rabbit antiserum to human placental SF (1:1000) (5) (*A*, *C*, and *D*) or nonimmune rabbit serum (1:1000) (*B*) as a negative control. Epidermal keratinocytes (Ep), dermal papillae (DP), and blood vessels (BV) are indicated. (Bar = 100 μm.)

Agreement between the *in vivo* and *in vitro* actions of angiogenic cytokines is not universal. Tumor necrosis factor (TNF) is angiogenic *in vivo* but *inhibits* endothelial cell proliferation *in vitro* (29, 30). Transforming growth factor β (TGFβ) is angiogenic *in vivo* (31) but inhibits endothelial motility and proliferation *in vitro* (17, 32, 33). Interleukin 6 (IL-6), a cytokine associated with tissue response to injury, is expressed in rodent endothelium transiently during physiologic angiogenesis (34). IL-6 also inhibits endothelial proliferation (35, 36). The early stages of capillary formation occur *without* endothelial proliferation in the irradiated rabbit cornea (37). Cytokines that trigger or participate in angiogenesis but inhibit endothelial growth may act to limit the spatial and temporal extent of vessel formation during physiologic angiogenesis. Furthermore, responses to these cytokines are modulated by the extracellular environment. Thus, TGFβ induces capillary-like tube formation in endothelial cells cultured within a collagen matrix (38). TNF and IL-6 each stimulate endothelial cell migration, and a combination of TNF and IL-6 stimulates migration significantly more than either agent alone (29, 36). TGFβ inhibits SF-stimulated migration (17). TNF induces endothelial cells to produce IL-6 (35). It is likely that coordinated expression of multiple interacting cytokines is required for self-limited physiologic angiogenesis.

Growth factors [TGFβ, FGF, and platelet-derived growth factor (PDGF)] are present in Matrigel and in the matrices of several tissues, including the cornea. We found that combinations of SF and either TGFβ, basic FGF, or PDGF gave greater stimulation of endothelial tube formation *in vitro* than did the same agents used individually (unpublished results). The concentrations studied (1 ng/ml) were about 10 times those found in 250 μg of Matrigel (39), and SF strongly stimulated tube formation on its own, by up to 8 times control. Nonetheless, SF may interact with other soluble mediators in our *in vivo* models. Our findings do not rule out the possibility that inflammatory cells contribute to the angiogenic response at high doses of SF. Angiogenic cytokines (e.g., TGFβ) can act as attractants for monocytes. TNFα is, in part, responsible for monocyte-mediated angiogenesis (29). It is not known if SF recruits monocytes or

modulates monocyte function. These considerations suggest that the angiogenic activity of SF may be multifactorial.

The major SF producer cells are fibroblasts, smooth muscle cells, and leukocytes. With rare exceptions (40), responder cells (epithelium, endothelium, melanocytes) are nonproducers (2, 3). The immunohistochemical studies of psoriatic plaques suggest that SF is produced by cells located outside of the blood vessel wall. Studies from our laboratory indicate that cultured endothelial cells express c-met mRNA and that immunoreactive c-met protein is present in blood vessel wall cells (endothelium and pericytes) in psoriatic plaques (unpublished results). We suggest that SF may play a role in microvessel formation or elongation in psoriasis and that its likely mode of action is paracrine.

SF (HGF) stimulates motility, invasiveness, proliferation, and morphogenesis of epithelium, and it may be involved in physiologic and pathologic processes such as embryogenesis (8, 41), wound healing (2, 13, 42), organ regeneration (43), inflammation, and tumor invasion (12, 42). Angiogenesis is a component of each of these processes. Therefore, the *in vivo* biologic action of SF may be due, in part, to its effects on both epithelial and vascular endothelial cells.

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