Regulation of Scatter Factor Production via a Soluble Inducing Factor

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Abstract. Scatter factor (SF) (also known as hepatocyte growth factor [HGF]) is a fibroblast-derived cytokine that stimulates motility, proliferation, and morphogenesis of epithelia. SF may play major roles in development, repair, and carcinogenesis. However, the physiologic signals that regulate its production are not well delineated. We found that various human tumor cell lines that do not produce SF secrete factors that stimulate SF production by fibroblasts, suggesting a paracrine mechanism for regulation of SF production. Conditioned medium from these cell lines contained two distinct scatter factor-inducing factor SF-IF activities: a high molecular weight (>30 kD), heat sensitive activity and a low molecular weight (<30 kD) heat stable activity. Further studies revealed that SF-producing fibroblasts also secrete factors that stimulate their own SF production. We characterized the <30-kD SF-IF activity from *ras*-3T3 (clone D4), a mouse cell line that overproduces both SF and SF-IF. The <30-kD filtrate from *ras*-3T3 conditioned medium induced four- to sixfold increases in expression of SF biologic activity, immunoreactive protein, and mRNA by multiple SF-producing fibroblast lines. *Ras*-3T3 SF-IF activity was stable to boiling, extremes of pH, and reductive alkylation, but was destroyed by proteases. We purified *ras*-3T3 SF-IF about 10,000-fold from serum-free conditioned medium by a combination of ultrafiltration, cation exchange chromatography, and reverse phase chromatography. The purified protein exhibited electrophoretic mobility of about 12 kD (reduced) and 14 kD (nonreduced) by SDS-PAGE. The identity of the protein was verified by elution of biologic activity from gel slices. Purified SF-IF stimulated SF production in a physiologic concentration range (about 20–400 *pM*). Its properties and activities were distinct from those of IL-1 and TNF, two known inducers of SF production. We suggest that SF-IF is a physiologic regulator of SF production.

SCATTER factor (SF)[1] was characterized as a cytokine secreted by stromal cell types that causes normally cohesive colonies of epithelial cells to spread out and to separate into individual cells (Stoker and Perryman, 1985; Stoker et al., 1987; Rosen et al., 1989). SF is now known to be identical to hepatocyte growth factor (HGF) (Weidner et al., 1991; Bhargava et al., 1992; Furlong et al., 1991), a serum-derived mitogen for mature rat hepatocytes (Miyazawa et al., 1989; Nakamura et al., 1989; Zarnegar and Michalopoulos, 1989). SF is a basic heparin-binding glycoprotein consisting of a heavy (60 kD) and a light (30 kD) chain (Gherardi et al., 1989; Rosen et al., 1989, 1990*a*; Weidner et al., 1990; Rubin et al., 1991). SF is a member of the family of kringle proteins. It exhibits similar subunit structure and 38% amino acid sequence identity to the proenzyme plasminogen (Nakamura et al., 1989). However, SF lacks proteolytic activity due to two critical amino acid substitutions at the catalytic center of its light chain. The cell surface receptor for SF was identified as the protein product of the *c-met* proto-oncogene, a 190-kD transmembrane tyrosine kinase (Bottaro et al., 1991; Naldini et al., 1991).

Recent studies suggest that SF may play significant roles in embryogenesis (Sonnenberg et al., 1993); morphogenesis of epithelia tissue (Montesano et al., 1991; Tsarfaty et al., 1992); blood vessel formation (Bussolino et al., 1992; Grant et al., 1993); organ regeneration (Matsumoto and Nakamura, 1993; Tsubouchi et al., 1993); and tumor cell dissemination (Weidner et al., 1990; Rosen et al., 1990*b*, 1991, 1994). Although some epithelial cell lines produce modest

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1. *Abbreviations used in this paper*: CM, Conditioned medium; ECL, enhanced chemiluminescence; HGF, hepatocyte growth factor; RPC, reverse phase chromatography; SF, scatter factor; SF-IF, SF-inducing factor; TFA, trifluoroacetic acid.

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quantities of SF (Adams et al., 1991; Tsao et al., 1993), SF's primary mode of action is thought to be paracrine. The major producer cell types (e.g., fibroblasts, smooth muscle cells, macrophages) are distinct from the responder cell types (epithelium, endothelium, melanocytes) (Stoker et al., 1987; Rosen et al., 1989, 1990*a*; Rubin et al., 1991). To further understand how SF functions in vivo, we have studied the factors that regulate its synthesis and secretion. We now report that SF production by fibroblasts and other stromal cell types is regulated, in part, by autocrine and paracrine mechanisms involving the accumulation of soluble scatter factor-inducing factors (SF-IFs). We characterized one such factor from an immortal fibroblast line that overproduces both SF and SF-IF.

Materials and Methods

Cell Types and Sources

Ras-transformed NIH/2-3T3 mouse fibroblasts (clone D4) (Stoker et al., 1987) were obtained from Dr. M. Stoker. MDCK cells were obtained from Dr. S. Warren (Yale University School of Medicine, New Haven, CT). Human fibroblast (CCD11Lu, WI38, CCD32Lu, CCL33Lu, MRC5, CCL215, 3ASubE), mouse fibroblast (BALB/c-3T3, Swiss albino 3T3), human squamous carcinoma (A253, Calul), human glioblastoma (U87MG, Hs683), and human mammary carcinoma (SKBr3, MCF7, BT549, MDA23, MDA468, Hs578T, T47D) cell lines were purchased from the American Type Culture Collection (Rockville, MD). Human mammary tumor-derived fibroblasts (1136TF) were provided by Dr. H. Smith (Geraldine Brush Cancer Research Institute, San Francisco, CA). Early passage human mammary tumor cell strains (173T, 186TL) were provided by Dr. P. Stampfer (Lawrence Berkeley Laboratory, University of California, Berkeley, CA). Human ovary carcinoma (YaOvBix2NMA, SKOV3, HEY) and mouse macrophage (Bac2F5) cell lines were provided by Dr. B. Kacinski (Yale University School of Medicine). Human melanoma cell lines (WW943, MeWo, Zazzaro) were provided by Dr. R. Halaban (Yale University School of Medicine). C2 rat hepatoma cells were provided by Dr. M. Weiss (Pasteur Institute, Paris, France). Bovine pericyte conditioned medium (CM) was provided by Dr. P. D'Amore (Children's Hospital Medical Center, Boston, MA). Human macrophage CM was provided by Dr. O. Einnarson (Yale University School of Medicine).

Cell Culture

Cells were grown in DME plus 10% (vol/vol) fetal calf serum (5% for *ras*-3T3 cells), 0.1 mM nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 μg/ml), as described earlier (Rosen et al., 1990*a*), with the following exceptions. Bac2F5 cells were grown in Iscove's medium supplemented with 10% fetal calf serum and 100 ng/ml colony stimulating factor-1 (CSF-1); and 173T and 186TL breast carcinoma cells were cultured in a defined medium based on MCDB 170, as described before (Stampfer et al., 1980). Cells were subcultured at weekly intervals using trypsin; reseeded at 1:4 (human lung fibroblasts, 1136TF) or 1:10 (all other cell types); and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Fibroblasts were studied within two to four passages after thawing of ampules.

Assay of CM for SF-inducing Activity

To generate CM, confluent cultures of cells were washed three times and incubated in serum-free DME (0.1 ml/cm^2) for 48 h. The medium was centrifuged to remove debris and stored at −20°C. The ability of the CM to stimulate SF production by MRC5 or CCD11Lu fibroblasts was assessed, as follows. Confluent fibroblasts in 2-cm^2 culture wells were washed and incubated in 0.5 ml of DME containing various dilutions of CM for 48 h. The resulting media were collected for SF assay, and the cells were counted using a Coulter counter. SF was quantitated by bioassay or ELISA. The SF production rate was expressed as MDCK scatter units or ng of SF/10^6 cells/48 h. For each assay condition, two to four replicate determinations of SF and cell number were made. Each experiment was performed at least three times.

Scatter Factor Bioassay

SF activity was quantitated using the MDCK serial dilution scatter assay, as described before (Rosen et al., 1989, 1990*a*). The SF concentration at the limiting dilution was defined as 0.5 MDCK scatter U/ml, allowing calculation of the SF titer in the original sample.

Scatter Factor ELISA

Immulon II 96-well plates (Dynatech Laboratories, Alexandria, VA) were coated with anti-human SF mouse monoclonal 23C2 ascites (Bhargava et al., 1992) (1:1,000) in 0.1 M Na_2CO_3 buffer, pH 9.6, overnight at 4°C (100 μl/well). Wells were washed four times with tris-buffered saline (20 mM tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) (TBS), blocked with 0.5% gelatin in TBS for 1 h at 37°C; washed four times, incubated for 1 h at 37°C with 100 μl of test sample or standard (recombinant human SF; Genentech, Inc., San Francisco, CA), and re-washed. Wells were then incubated with rabbit anti-SF antiserum (Grant et al., 1993) (1:2,000) for 1 h at 37°C in 0.25% gelatin in TBS, washed four times, incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (50 μg/ml) (1 h at 37°C), and washed again. Color was developed by incubation with 100 μl of substrate solution (*p*-nitro phenyl phosphate [1 mg/ml], 1 M diethanolamine HCl, 2 mM $MgCl_2$, pH 9.8). The reaction was stopped by adding 50 μl of 3 M NaOH, and absorbance was read at 410 nm using a Dynatech 96-well spectrophotometer. The assay was specific for SF. It did not detect plasminogen, albumin, or various other growth factors and cytokines. The standard curve was linear from 0.2–4.0 ng/ml of SF (useful measuring range).

Northern Blotting

Confluent fibroblasts in 100 mm dishes (10 ml DME/dish) were exposed to various treatments, and total cell RNA was isolated by acid guanidinium isothiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Aliquots of RNA (20 μg/lane) were electrophoresed through 1% agarose–17% formaldehyde gels, and RNA quality and loading were assessed by ethidium bromide staining. Northern transfers and hybridization were performed as described before (Elias and Lentz, 1990). Membranes were probed with a 2.2-kb insert containing the complete human HFG cDNA subcloned into the SalI restriction site of the pUC vector. (The insert was subcloned from the EMC-hHGF construct [Nakamura et al., 1989], provided by Dr. T. Nakamura). This probe will detect all SF mRNA transcripts, including the major 6-kb mRNA, the 3-kb mRNA (which also encodes full-length protein), the 1.5-kb RNA (which encodes a truncated form of SF), and other mRNAs (four 2.2 kb) that have not been fully characterized (Rubin et al., 1991; Chen et al., 1991). Sizes of transcripts were assessed by comparison to RNA size standards (Bio-Rad Laboratories, Richmond, VA). A cDNA encoding the 28S ribosomal gene (Erikson et al., 1981) was used as a control probe. (The 28S cDNA was provided by Dr. S. Adams, University of Pennsylvania, Philadelphia, PA). cDNA probes were labeled by nick translation to specific activities of ~10^9 CPM/μg DNA. Membranes were washed with 0.5× SSC containing 0.1% SDS for 30 min at 50°C, and autoradiography was performed. Densitometry was carried out using a Hoeffer GS370 densitometer, and data were analyzed using the GS300 software package.

Western Blotting

CM samples were concentrated 20-fold by ultrafiltration using YM-30 membrane (Amicon Corp., Danvers, MA). Aliquots (75 μl) were electrophoresed under nonreducing conditions and blotted, using rabbit anti-SF antiserum (1:2,000) as the primary antibody, as described before (Bhargava et al., 1992). Bound anti-SF antibody was detected by enhanced chemiluminescence (ECL) (Amersham Corp., Arlington Heights, IL).

Interleukin-1 Preparations and IL-1 Bioassay

Recombinant human interleukin-1 (IL-1) α and IL-1β were a gift from Dr. R. Drummond (Cetus Corporation, Emeryville, CA). Recombinant murine IL-1α was a gift from Dr. P. Lomedico (Hoffman-La Roche). The levels of IL-1 in CM were quantitated using a sensitive and specific assay, based on the ability of IL-1 in the samples to stimulate proliferation of D10.N(4)M cells, a subclone of the IL-1–dependent cloned murine T cell line D10.G4.1 (Hopkins and Humphreys, 1989). The assay detected ⩾0.8 ng/ml of recombinant mouse IL-1α.

Table I. Stimulation of MRC5 SF Production by CM from Human Breast Carcinoma Cells

Cm tested*	MRC5 SF production
	*(ng/10⁶ cells/24 h)**
Control (0)	4
SKBr3	10
MCF7	9
BT549	19
MDA23	25
MDA468	26
Hs578T	6
T47D	14
173T	11
186TL	13
‡*ras*-3T3 (D4)	24

* Confluent tumor cells were incubated in DME (0.1 ml/cm^2) for 48 h to produce CM. To assay the CM for SF-inducing activity, confluent MRC5 fibroblasts in 2-cm^2 wells were incubated in 0.5 ml DME containing 50% CM for 24 h. The media were assayed for SF by ELISA. Values are means of two assays (range of ±15%).

‡ For *ras*-3T3 cells, which produce SF, the <30-kD filtrate of the CM was tested.

Purification of ras-3T3 SF-IF

SF-IF was purified from CM from *ras*-3T3 (D4), a mouse cell line that produces very high titers of SF (Stoker et al., 1987; Rosen et al., 1990*a*). Cells were grown to confluency in Falcon 1750-cm^2 plastic roller bottles in 300 ml DME plus 5% fetal calf serum. To generate CM, the cells were washed three times and incubated in serum-free DME (175 ml) for 2-3 d. CM was centrifuged to remove debris and stored at −20°C.

The CM was ultrafiltered using an Amicon YM-30 membrane, and the <30-kD filtrate was chromatographed on BioRex-79 (Bio-Rad Laboratories, Cambridge, MA), a weak cation exchanger. 700 ml of filtrate was applied to a 40 ml bed BioRex-70 column equilibrated in Tris buffer (20 mM, pH 7.5). The column was washed with 200 ml of buffer and eluted with 200 ml of 1.5 M NaCl in Tris buffer. The eluate was de-salted by dialysis, using a 1-kD membrane, and applied to a 10 ml bed (0.9 cm × 15 cm) BioRex-70 column. This column was washed with Tris buffer and eluted using a 30 ml NaCl gradient (0–1.5 M) at ~1.5 ml/min. 1-ml fractions were collected, and the fractions were dialyzed, lyophilized, re-hydrated, and assayed for SF-IF activity using MRC5 as target cells.

Active fractions were pooled and applied to an HR10/10 PepRPC FPLC column (8 ml bed volume) (Pharmacia Fine Chemicals, Piscataway, NJ) in a sample volume of 0.5 ml. The column was eluted with a 0–100% gradient of acetonitrile in water (containing 0.1 g/l of trifluoroacetic acid [TFA]) at 1.0 ml/min for 30 min, and 1.0 ml fractions were collected. Fractions were lyophilized, rehydrated, and assayed for SF-IF activity. Active fractions were re-chromatographed on the same column, yielding a single peak of absorbance. An aliquot of this peak was applied to a narrow-bore C18 HPLC column. This column was eluted with a gradient of acetonitrile in water (plus TFA), at a rate of ~1% per min. Peaks containing protein were lyophilized, rehydrated, and used for bioassays and SDS-PAGE analysis. Protein was assessed by absorbance at 280 nm (and at 214 nm at the final stage of purification), and by the Bio-Rad dye-binding microassay. Ultrafiltration and standard chromatography were performed at 4°C, while FPLC and HPLC were performed at 25°C. An aliquot of highly purified SF-IF from the C18 HPLC column was used for direct amino acid microsequencing. Sequence analysis was performed at the Laboratory for Molecular Analysis (Albert Einstein College of Medicine).

Elution of SF-IF Activity from Gel Slices

Ras-3T3 SF-IF partially purified on BioRex-70 (1.5 ml) was analyzed preparatively on a 15% SDS-polyacrylamide gel, using a slot width of 8 cm, without reduction. The unstained gel was sectioned into 4 mm slices, and each slice was crushed and eluted by passive diffusion for 48 h at 4°C in 2 ml of 3 mM NH_4HCO_3 buffer, pH 8. Eluates were dialyzed, dried, rehydrated in 50 μl of DME, and assayed for SF-IF activity using MRC5 cells.

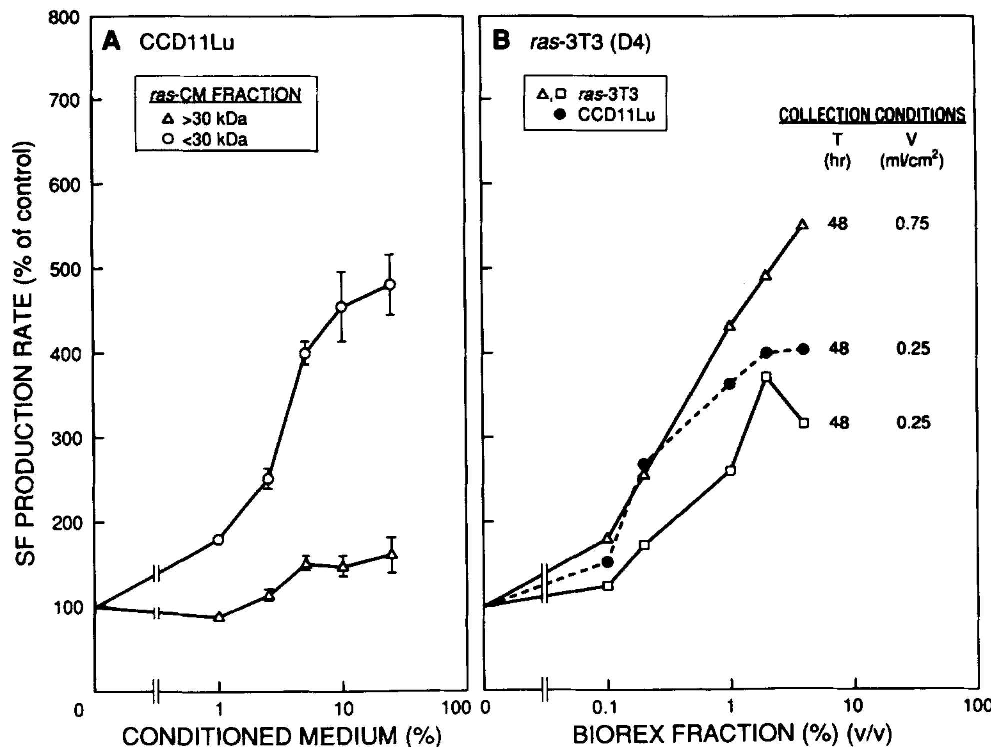

Figure 1. (*A*) Effects of different fractions of *ras*-3T3 CM on SF production by CCD11-Lu human fibroblasts. CM was chromatographed on S-Sepharose to remove SF, and the SF-free void was separated into >30- and <30-kD fractions by ultrafiltration. Fractions were assayed for stimulation of production of SF activity by CCD11Lu. Values are means ± SEMs of triplicate assays. (*B*) Stimulation of *ras*-3T3 SF production by partially purified *ras*-3T3 SF-IF. The *ras*-3T3 CM <30-kD filtrate was partially purified using BioRex-70 (see Materials and Methods). The BioRex-70 eluate was dialyzed, lyophilized, re-hydrated in DME, and assayed for stimulation of SF production by *ras*-3T3 cells. Two different collection volumes were used (see Figure). The response of CCD11Lu cells is shown for comparison. Values are means of duplicate assays, with ranges within ±15% of the means.

Table II. Properties of ras-3T3 SF-IF Activity

Treatment	SF-IF activity* (Percent of control)
Control (no treatment)	100
Ultrafiltration	
>30-kD retentate	4 ± 4
<30-kD filtrate	100 ± 12
Dialysis	
Undialyzed	100 ± 4
1-kD membrane	141 ± 47
5-kD membrane	80 ±
12–14-kD membrane	3 ± 3
25-kD membrane	0
Heat (100°C × 5 min)	126
pH (2 h at 25°C)	
1.0	57 ± 5
3.0	84 ± 6
7.0	100 ± 9
10.0	93 ± 7
12.0	96 ± 6
Proteases§	
Trypsin	0
Pronase	0
Reductive alkylation‖	100

* Activity is expressed relative to that of *ras*-3T3 CM <30-kD filtrate (*control*).
‡ *Ultrafiltration.* CM was separated into <30- and >30-kD fractions using an Amicon YM-30 filter. The >30-kD retentate was chromatographed on S-Sepharose to remove SF prior to assay for SF-IF.
§ *Protease treatment. Trypsin.* Samples were incubated with bovine trypsin (50 μg/ml) for 60 min at 37°C, and soybean trypsin inhibitor (100 μg/ml) was added. For controls, soybean trypsin inhibitor was added before trypsin. *Pronase.* Samples were concentrated 100-fold and incubated with 0.2 U/ml pronase (Sigma P-0652) for 24 h at 37°C. Pronase activity was stopped by dilution or by boiling for 5 min. Controls without pronase were treated identically.
‖ *Reductive alkylation.* SF-IF was incubated with DTT (5 mM) for 90 min at 37°C; and then with iodoacetamide (10 mM) for 60 min at 37°C. Factor was dialyzed using a 1-kD membrane, to remove DTT and iodoacetamide. Controls were incubated without DTT or iodoacetamide and dialyzed similarly.

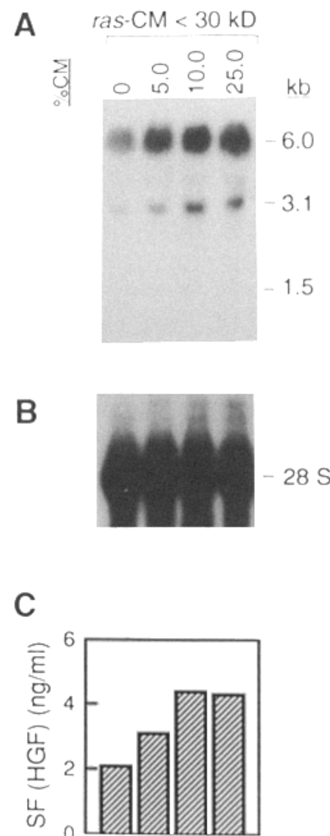

Figure 2. Stimulation of SF mRNA expression in CCD11-Lu fibroblast cultures by *ras*-3T3 CM <30 kDa filtrate. Confluent cultures were incubated for 24 hr with different concentrations of *ras*-3T3 filtrate. The total cell RNA was extracted, and equal aliquots of RNA (20 μg per lane) were electrophoresed and Northern blotted. Blots were probed using a cDNA for human SF (panel A). As a control, the blots were stripped and reprobed for 28S ribosomal RNA expression (panel B). The concentrations of SF in the culture medium were determined by ELISA (panel C).

Results

Stimulation of SF Production by Human Tumor Cell CM

We screened CM from multiple lines of breast carcinoma cells for SF-inducing activity (defined as the ability to stimulate human fibroblast SF production by more than twofold). None of the CM samples contained any SF detectable by bioassay or ELISA. However, eight out of nine CM samples contained SF-inducing activity (Table I). These CM samples induced the production of SF biologic activity, protein, and mRNA (see below). To further characterize the SF-inducing activity, CM samples were ultrafiltered using an Amicon YM-30 membrane. Both the <30-kD filtrate and the >30-kD retentate contained SF-inducing activity; but the relative proportions of these activities varied among different cell lines. The >30-kD SF-IF activity was completely destroyed by boiling (100°C × 5 min), while the <30-kD activity was heat stable (not shown). Both activities were abolished by treatment with trypsin. In addition to breast carcinoma cells, we detected high and low molecular weight SF-IF activities in CM from other tumor types (see below), suggesting that tumor cells produce at least two distinct SF-IF activities.

Stimulation of SF Production by Factors in ras-3T3 CM

Ras-3T3 (D4) is a line of mouse fibroblasts that produces extremely high titers of SF. While determining the optimal conditions for collection of *ras*-3T3 CM for purification of SF (Rosen et al., 1990*a*), we found that the total SF production was inversely related to the volume of collection medium. Similar relationships between SF production and volume were observed for three lines of normal human fibroblasts (CCD11Lu, WI38, CCL33Lu). SF production rates were (2.5–3)-fold greater at small volumes (0.1 ml/cm²) than at large volumes (≥0.5 ml/cm²). These results suggested that SF production by fibroblasts might be regulated, in part, by the accumulation of a soluble SF-inducing factor(s).

To determine if *ras*-3T3 CM contained SF-IF activity, SF was removed from the medium, selectively, by chromatography on S-Sepharose fast flow (Pharmacia Fine Chemicals), a resin with high affinity for SF (Rosen et al., 1990*a*). At a CM/bed volume ratio of 50:1, the S-Sepharose column bound all of the SF activity but only 1% of the total protein. No SF was found in the void fraction. The SF-free *ras*-3T3 void was filter-sterilized and assayed for SF-inducing activity. Addition of *ras*-3T3 void induced dose-dependent increases in SF production rate by multiple SF-producing fibroblasts lines (Fig. 1, and see below). To quantitate SF-IF activity, one SF-IF U/ml was defined as the dose of SF-IF needed to double the rate of SF production by human lung

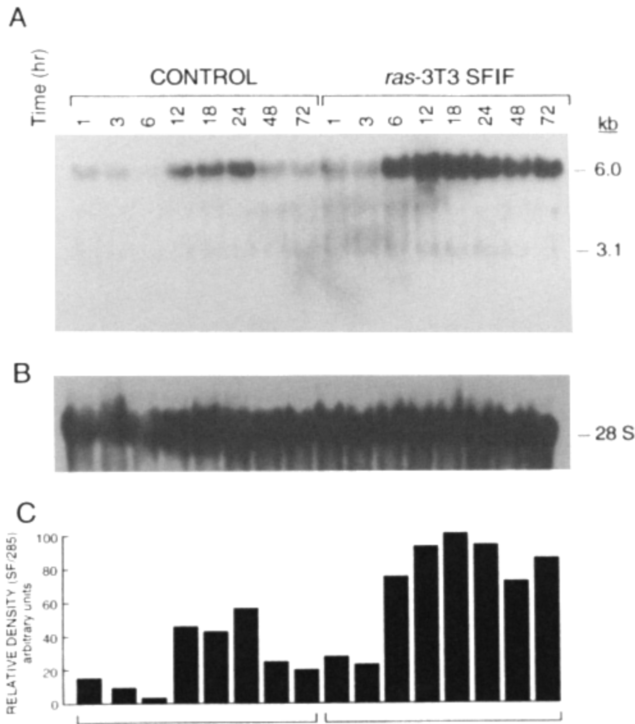

Figure 3. Time course of SF mRNA expression in CCD11Lu fibroblast cultures treated without or with *ras*-3T3 CM <30 kDa filtrate. Confluent CCD11Lu cultures were incubated for various times up to 72 h without (control) or with 10% (v/v) of *ras*-3T3 CM <30 kDa filtrate. the total cell RNA was extracted, electrophoresed (20 μg per lane), and Northern blotted. Panel A shows SF mRNA expression; panel B shows 28S ribosomal RNA expression; and panel C shows the densitometric ratios of SF(6 kb)/28S for control and *ras*-3T3 (SF-IF)-treated cells.

fibroblasts (CCD11Lu or MRC5). The titer of SF-IF activity in *ras*-3T3 CM S-Sepharose void (60–70 U/ml) was considerably higher than the titers found in human tumor cell CM (2–18 units/ml). Thus, *ras*-3T3 cells were utilized for further characterization of the SF-IF activity.

Properties of ras-3T3 SF-IF Activity

After fractionation using an Amicon YM-30 membrane, nearly all of the SF-IF activity was found in the <30-kD filtrate (Table II, Fig. 1 *A*). Subsequent studies were performed using the <30-kD filtrate without S-Sepharose chromatography, since no SF was present in these filtrates, and since they contained the same SF-IF activity as the S-Sepharose void. Most of the SF-IF activity was retained after dialysis using 1- and 5-kD membranes, whereas little or no activity was retained by 12–14 or 25-kD membranes (Table II). SF-IF activity was stable to boiling, reductive alkylation, and exposure to a wide range of pH values. Treatment with proteases (trypsin or pronase) destroyed the activity. These data suggest that the SF-IF activity is due to a stable, low molecular weight (5–15 kD) protein(s). There was no difference in SF-IF activity whether the assays were performed without or with serum. Neither crude CM nor highly purified *ras*-3T3 SF-IF had a significant effect on the fibroblast cell number. There was no evidence of target fibroblast toxicity, as judged by morphology or exclusion of trypan blue dye.

Sensitivity of Different Lines of Fibroblasts of SF-IF Activity

Six of six human lung fibroblast lines (CCD11Lu, MRC5, WI38, CCD32Lu, CCL33Lu, CCL215) were responsive to *ras*-3T3 CM <30-kD filtrate. The concentrations of filtrate (vol/vol) required to double the rate of SF production were generally similar (1.5–3%). The maximum stimulation of SF production, relative to control, was four- to sixfold. These maximum responses were observed at 10–25% filtrate. *Ras*-3T3 cells themselves were responsive to partially purified *ras*-3T3 SF-IF activity (BioRex-70 fraction) (Fig. 1 *B*). When assayed using the same collection volume (0.25 ml/cm^2), *ras*-3T3 cells were less sensitive to BioRex fraction than were CCD11Lu human fibroblasts (Fig. 1 *B*). However, increasing the collection volume from 0.25 to 0.75 ml/cm^2 resulted in increased sensitivity of *ras*-3T3 cells to BioRex fraction. The doses of BioRex fraction required to double the SF production rate were 0.10, 0.13, and 0.47% (vol/vol), respectively, for *ras*-3T3 (V = 0.75 ml/cm^2), CCD11Lu (0.25 ml/cm^2), and *ras*-3T3 (0.25 ml/cm^2). The apparent reduced sensitivity of *ras*-3T3 cells to exogenous factor at the smaller collection volume may reflect the high rate of endogenous production of SF-IF activity by these cells.

Effect of SF-IF Activity on SF mRNA Expression

Treatment of human fibroblasts with *ras*-3T3 CM <30-kD filtrate resulted in coordinate increases in expression of all SF mRNA species, although in most experiments, only the more abundant 6- and 3-kb mRNs were visualized well. Treatment of CCD11Lu cells for 24 h with *ras*-3T3 filtrate induced a dose-dependent increase in the SF mRNA expression, with maximal responses at 10–25% filtrate (vol/vol) in different experiments (Fig. 2 *A*). Corresponding increases in SF protein, as determined by ELISA, were also observed (Fig. 2 *C*). SF mRNA expression increased within 6 h after addition of *ras*-3T3 filtrate, peaked at 18 h, and then declined slowly, but remained above control levels at 72 h (Fig. 3 *A*). Control cultures also showed time-dependent increases in SF mRNA expression that were smaller in magnitude than for treated cultures (Fig. 3 *A*). Similar time-dependent changes in SF mRNA expression in control cultures were observed in other experiments; these changes may be due, in part, to an endogenous SF inducer (see below). After 48–72 h, expression of the 6-kb SF mRNA was about three- to fourfold higher in treated cultures than in control cultures (Fig. 3 *C*). These increases in SF mRNA expression correlated with four- to sixfold increases in SF protein production, determined by ELISA (data not shown). Similar to *ras*-3T3 CM, media from various lines of human mammary carcinoma cells (see Table I) also stimulated SF mRNA expression. SF itself (20 ng/ml) did not affect SF mRNA expression in CCD11Lu fibroblasts (not shown).

SF-IF Producer and Responder Cell Types

CM from six of six lines of SF-producing human fibroblasts contained <30-kD SF-IF-like activity. However, the activity titers (4–10 U/ml) were much less than that in *ras*-3T3 CM (60–70 U/ml). Three lines of non-SF–producing fibroblasts

*Table III. Cell Types That Produce and Respond to SF-IF**

Cell line	Cell type	SF production	SF-IF production	SF-IF response
Ras-3T3 (D4)	Mouse fibroblast	+	+	+
CCD11Lu	Human lung fibroblast	+	+	+
CCD32Lu	"	+	+	+
CCL33Lu	"	+	+	+
WI38	"	+	+	+
CCL215	"	+	+	+
MRC5	"	+	+	+
3AsubE SV40 tx	Human placenta fibroblast	−	−	−
BALB/c-3T3	Mouse fibroblast	−	−	−
Swiss albino 3T3	"	−	−	−
Bac2F5	Mouse macrophage	+	−	−
Human macrophage		+	−	−
RASM	Rat aorta smooth muscle	+	−	+
U87MG	Human glioblastoma	+	+	+
Hs683	"	+	+	+
Bovine pericyte		−	−	−
C2	Rat hepatoma	−	−	−
SKBr3	Human mammary carcinoma	−	+	−
MCF7	"	−	+	−
BT549	"	−	+	−
MDA23	"	−	+	−
MDA468	"	−	+	−
Hs578T	"	−	−	−
T47D	"	−	+	−
173T	"	−	+	−
186TL	"	−	+	−
YaOvBix2NMA	Human ovary carcinoma	−	−	−
SKOV3	"	−	−	−
HEY	"	−	−	−
MDCK	Canine kidney epithelium	−	−	−
A253	Human squamous carcinoma	−	−	−
Calu1	"	−	−	−
WW943	Human melanoma	−	−	−
MeWo	"	−	−	−
Zazzaro	"	−	−	−

* To assay for SF-IF production, confluent test cells were incubated with serum-free DME (0.1 ml/cm²) for 48 h to generate CM. The CM was assayed for its ability to stimulate SF production by human lung fibroblasts. For cell types that produce SF, the <30-kD filtrate of the CM was assayed. (+) Greater than twofold stimulation of SF production. (−) denotes no stimulation of SF production at up to 50% test CM (i.e., <2 SF-IF units/ml). To assay for response to SF-IF, confluent test cells were incubated with different concentrations of *ras*-3T3 <30-kD filtrate for 48 h in 2 cm² wells (0.5 ml of DME per well), and the SF production rates were determined. (+) Greater than twofold stimulation of SF production by the test cells. (−) No response at up to 25% ras-3T3 <30-kD filtrate (15 U/ml of SF-IF).

(BALB/c-3T3, Swiss albino 3T3, 3ASubE) did not produce any detectable SF-IF activity. In addition to breast carcinoma cells, two lines of human glioblastoma cells produced SF-IF activity. On the other hand, no SF-inducing activity was found in CM from macrophages, bovine pericytes, rat hepatoma cells, MDCK epithelial cells, two lines of human squamous carcinoma cells, three lines of ovary carcinoma cells, and three lines of melanoma cells. Seven of seven SF-producing lines of fibroblasts responded to *ras*-3T3 CM. In contrast, multiple non-SF–producing lines of fibroblasts, epithelial cells, and tumor cells did not produce detectable levels of SF in response to *ras*-3T3 CM (Table III). The responses of various stromal cell types to purified SF-IF is described below.

Ras-3T3 SF-IF Activity Is Distinct from IL-1

IL-1 was reported to stimulate SF production by human dermal and lung fibroblasts (Matsumoto et al., 1992*a*; Tamura et al., 1993). We found that recombinant murine IL-1α, human IL-1α, and human IL-1β each stimulated production of SF activity, protein, and mRNA by CCD11Lu cells. A doubling of SF production required ~0.1–0.2 ng/ml, while maximal (fourfold) stimulation of SF production required ⩾2 ng/ml of IL-1 (not shown). We used a sensitive bioassay to quantitate IL-1 biologic activity (see Materials and Methods). *Ras*-3T3 S-sepharose void and *ras*-3T3 CM <30-kD filtrate, which contained 60–70 U/ml of SF-inducing activity, had no detectable IL-1 activity. The BioRex-70 fraction, which contained 1,000 U/ml of SF-IF activity, had IL-1-like activity. The BioRex-70 fraction, which contained 1,000 U/ml of SF-IF activity, had IL-1-like activity corresponding to only 12 pg/ml of recombinant mouse IL-1α. Further evidence that SF-IF and IL-1 are distinct is presented below.

Effects of Other Cytokines and Growth Factors on SF Production

We screened a variety of factors for SF-inducing activity using various SF-producing human cell types as target cells, including MRC5 lung fibroblasts, mammary tumor-derived fibroblasts (1136TF), and glioblastoma cells (U87MG, Hs683) (Table IV). Recombinant human IL-1α, human IL-1β, mouse IL-1α, and human TNFα gave maximal stimula-

Table IV. Responses of Various Stromal Cell Types (MRC5 Human Lung Fibroblasts, U87MG, and Hs683 Human Glioblastoma Cells, 1136TF Human Mammary Tumor-derived Fibroblasts) to Purified SF-IF and to Various Other Cytokines*

Agent		SF production rate*			
		MRC5	U87MG	Hs683	1136TF
	(ng/ml)	*(ng/10^6 cells/24 h)*			
Control	(0)	5.1	1.0	5.0	2.4
ras-3T3 SF-IF	(5)	25.0	4.5	11.8	12.6
IL-1α	(5)	14.1	0.7	5.8	2.7
IL-1β	(5)	11.2	1.0	4.8	2.3
IL-6	(20)	5.0	0.9	4.7	2.1
IL8	(20)	7.0	1.0	4.5	2.2
IL11	(20)	5.8	NT	NT	2.5
TNFα	(20)	13.7	1.3	3.1	2.2
Oncostatin-M	(20)	6.9	1.4	4.1	3.4
Basic FGF	(20)	8.7	0.9	3.7	1.9
EGF	(20)	6.0	0.8	6.3	2.8

* *Ras*-3T3 SF-IF was purified as described in the text. Confluent target cells in 2-cm^2 wells were incubated in 0.5 ml of serum-free DME containing various agents for 48 h, and SF was measured by ELISA. Values are averages of two assays (range ±15% of the mean).

tion of SF production by MRC5 cells at concentrations of 5, 5, 5, and 20 ng/ml, respectively. However, neither of these agents alone or in combination stimulated SF production by U87MG, Hs683, or 1136TF (Table IV). EGF, basic FGF, PDGF, oncostatin-M, and various interleukins and interferons did not stimulate SF production by any of these cell types. In a separate set of experiments, we tested the ability of various factors to stimulate SF production by CCD11Lu human lung fibroblasts. Each agent was studied over a range of concentrations (up to 20 ng/ml for purified cytokines). The following agents had no effect on SF production: EGF, TGFα, PDGF, basic FGF, bovine pituitary extract (which contains a mixture of acidic and basic FGFs), keratinocyte growth factor, mast cell growth factor, CSF-1, vascular endothelial cell growth factor, oncostatin-M, lipopolysaccharide (endotoxin), interferon-γ, various interleukins (IL-2, IL-4, IL-6, IL-7, IL-8, IL-11), fetal calf serum, and calf serum. TNFα induced a small increase in SF production but did not stimulate SF mRNA expression in CCD11Lu. TGFβ, a known inhibitor of SF expression (Gohda et al., 1992), blocked basal and *ras*-3T3 CM-stimulated SF production.

Purification of SF-IF

We purified SF-IF from *ras*-3T3 CM using a combination of ultrafiltration, cation exchange chromatography, and reverse phase chromatography (RPC) (Fig. 4 and Table V). Further chromatography of the active PepRPC fractions (see Fig. 4 *B*) on a C18 HPLC column revealed one major protein peak containing all of the SF-IF activity (Fig. 5). Highly purified SF-IF from PepRPC columns exhibited relative molecular mass values of 14 ± 2 kD under nonreducing conditions and 12 ± 2 kD under reducing conditions on silver-stained SDS-polyacrylamide gels (Fig. 6). SDS-PAGE analysis of C18 HPLC-purified SF-IF revealed the same band (not shown). The identity of this protein was verified by elution of SF-IF activity from SDS-polyacrylamide gel slices. Nearly all of the activity recovered from the gel was found in the gel slice corresponding to about 14 kD (nonreduced). A dose-response curve for the purified factor is presented in Fig. 4 *B* (*inset*). Based on these and other dose-response data and the relative molecular mass of the purified factor, as determined by SDS-PAGE, we estimate that twofold stimulation of MRC5 SF production requires about 15-25 pM of SF-IF, and maximal stimulation of SF production requires 300-500 pM of SF-IF. Highly purified *ras*-3T3 SF-IF stimulated SF by human lung fibroblasts, mammary fibroblasts, and glial cells, as demonstrated by ELISA (Table IV) and Western blotting (Fig. 7).

Direct sequencing of an aliquot of purified SF-IF from the HPLC column revealed a single NH_2-terminal sequence: AKNDAIKIGA. This sequence did not match or show close homology with any sequences in the Protein Identification Resource, Swiss-Prot, GeneBank, or GenEMBL databases, suggesting that SF-IF is a new and unique protein.

Discussion

We have presented evidence that autocrine and paracrine mechanisms involving soluble-inducing factors regulate SF production by fibroblasts and other stromal cell types. We have provided a detailed characterization of one such factor produced by a line of *ras*-transformed mouse 3T3 cells that overexpresses SF. This SF-inducing factor is a heat stable protein with a molecular size of about 12 ± 2 kD (reduced) by SDS-PAGE with silver staining. SF-IF activity was not detected in fetal calf serum; and the production of and response to SF-IF did not require serum.

*Table V. Purification of ras-3T3 SF-IF**

Step	Activity	Protein	Spec. Act.	Fold purification	Recovery
	(U)	*(μg)*	*(U/μg)*		*(%)*
Ras-3T3 CM (700 ml)	46,000	84,000	0.5	1	100
Ultrafiltration	46,000	28,000	1.6	3	100
1st BioRex column	33,600	840	40	80	73
2nd BioRex column	28,000	165	170	340	61
PepRPC (C2/18 FPLC)	15,000	5	3,000	6,000	33
HPLC (C18)‡	1,500	0.31	4,840	9,680	–

* SF-IF was purified as described in the Materials and Methods section. Chromatographic fractions were dialyzed, lyophilized, rehydrated, and assayed for SF-IF activity using MRC5 fibroblasts.
‡ An aliquot of the FPLC peak containing biologic activity was subjected to chromatography on a narrow-bore C18 HPLC column.

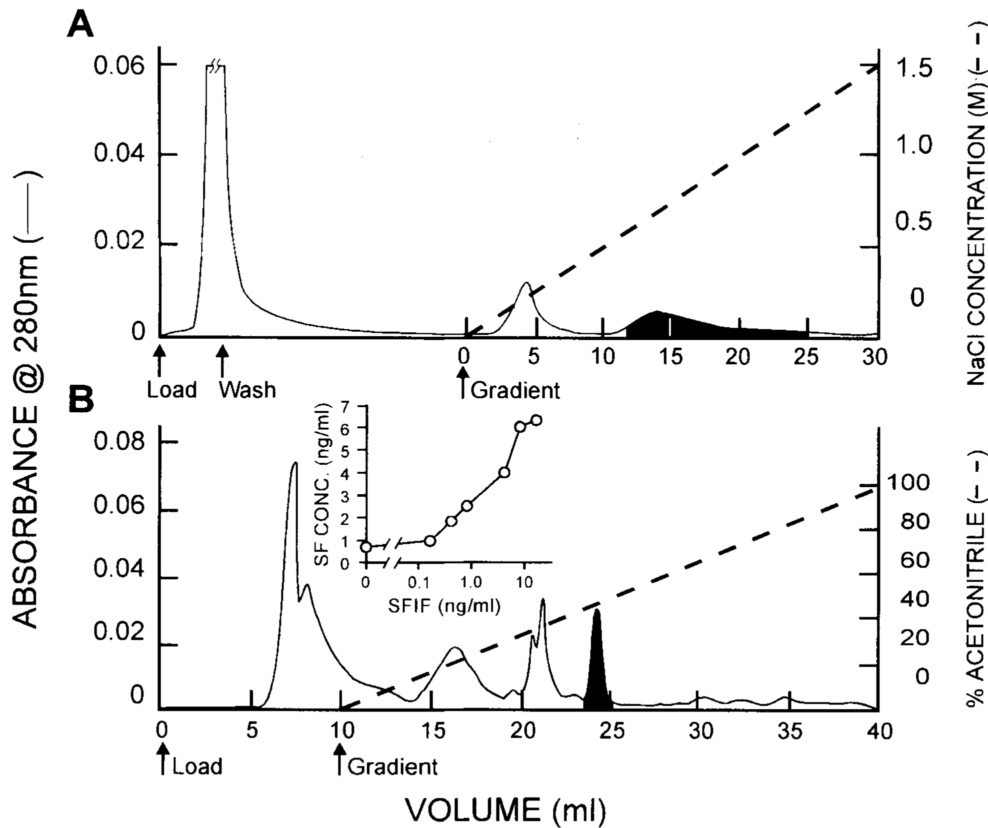

Figure 4. Purification of *ras*-3T3 SF-IF. The *ras*-3T3 CM <30 kDa filtrate (700 ml) was chromatographed twice on BioRex-70. The second BioRex-70 column was eluted with an NaCl gradient (panel *A*). Fractions containing SF-IF activity (shaded area) were pooled and applied to a reverse phase FPLC (PepRPC) column (panel *B*). RPC fractions were dried, rehydrated in 50 μl of DMEM, and assayed for biologic activity. All of the *SF-IF* activity was eluted in a small peak at about 45% acetonitrile (shaded area). The inset shows a dose-response curve for stimulation of MRC5 SF production by purified SF-IF.

We found that human mammary tumor and glioblastoma cells produce soluble high and low molecular weight SF-inducing factors. Seslar and colleagues (1993) reported that MCF7 breast carcinoma cells secrete an activity that stimulates SF protein and mRNA expression by MRC5 fibroblasts. This activity was >30 kD and heat sensitive, similar to the high molecular weight inducer that we detected, but distinct from the factor that we purified. *Ras*-3T3 SF-IF stimulated expression of SF in several stromal cell types, including lung fibroblasts, mammary tumor fibroblasts, and glial cells. Thus, stromal cells stimulated by SF-IFs may be a rich source of SF production within tumors. This hypothesis is supported by the finding that large quantities of both SF and SF-inducing activity are present in extracts of solid EMT6 mouse mammary tumors (Rosen et al., 1994). Cultured EMT6 cells did not produce SF, but did produce an SF-IF-like activity for fibroblasts. Interactions between epithelial and stromal cells are thought to regulate the expression of urokinase and metalloproteinases, enzymes that potentiate tumor cell invasion (van den Hooff, 1988). SF is a very potent stimulator of tumor cell motility, protease expression, and invasion (Rosen et al., 1990*b*, 1991; Weidner et al., 1990;

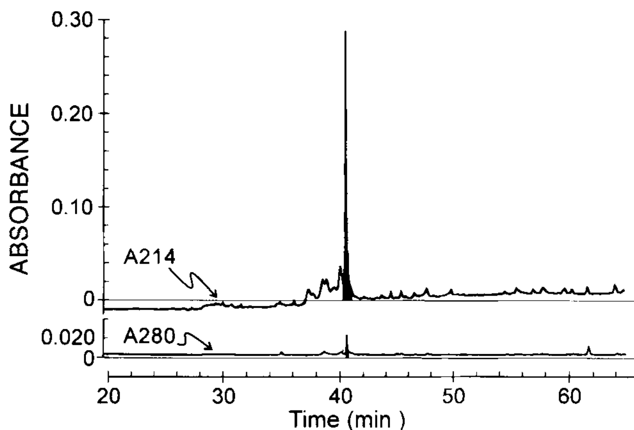

Figure 5. C18 reverse phase HPLC of SF-IF. An aliquot of SF-IF from the PepRPC column (Fig. 4 *B*) was analyzed on a narrow-bore C18 HPLC column. The major peak, which eluted at 41% acetonitrile, contained SF-IF biologic activity (shaded area). None of the minor peaks contained detectable activity.

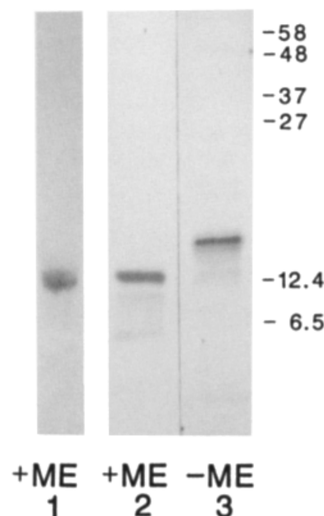

Figure 6. SDS-PAGE of highly purified SF-IF. SF-IF purified from *ras*-3T3 CM (PepRPC; Fig. 4 *B*) was analyzed on a silver-stained 15% SDS-polyacrylamide gel with [+ME (mercaptoethanol)] and without (−ME) reduction. Lane 1 and lanes (2+3) show SF-IF from two different purifications. The positions of the reduced molecular weight standards are indicated on the right.

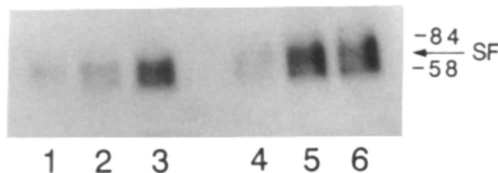

Figure 7. Western blotting of CM from SF-producing cells treated with purified SF-IF. Confluent U87MG glioblastoma cells and MRC5 fibroblasts were incubated in DMEM (10 ml/100 mm dish) ± SF-IF for 24 h. The CM was concentrated and blotted to detect SF. Lanes *1–3* represent U87MG; lanes *4–6* represent MRC5. The doses of SF-IF were 0 (lanes *1* and *4*), 1.2 ng/ml (lanes *2* and *5*), and 5 ng/ml (lanes *3* and *6*). The arrow shows the position of recombinant human SF.

Rosen et al., 1994). SF is also a potent mediator of pathologic angiogenesis (Grant et al., 1993; Naidu et al., 1994). Our studies provide a further mechanism by which tumor:stroma interactions may contribute to malignant growth.

IL-1 and TNF, two multifunctional cytokines associated with tissue response to injury, were reported to stimulate SF production by some human fibroblast lines (Matsumoto et al., 1992*a*; Tamura et al., 1993). We observed increases in SF protein and mRNA expression in multiple IL-1–treated human lung fibroblast lines (unpublished results). However, IL-1, TNF, and combinations of IL-1 plus TNF did not stimulate SF production in several other stormal cell types responsive to purified SF-IF (Table IV). Using a sensitive bioassay of IL-1, crude preparations of *ras*-3T3 CM, which contained large amounts of SF-IF activity, contained little or no IL-1. Partially purified SF-IF (BioRex fraction) contained the equivalent of 12 pg/ml of IL-1. Based on the ability of IL-1 to stimulate MRC5 SF production, the amount of IL-1 needed to account for the SF-IF activity in the BioRex fraction would be about 1,000 SF-IF U/ml × (0.1–0.2 ng/ml)/SF-IF U = 100–200 ng/ml (8,300–16,600 times the measured quantity). This calculation is subject to uncertainty due to the potential influence of other inhibitory or synergistic molecules in the preparation. Nonetheless, it suggests that IL-1 can account for only a small fraction of the SF-IF activity produced by *ras*-3T3 cells. Finally, the aminoterminal sequence of purified SF-IF did not match any known protein. SF-IF may be a prototype of a specific class of physiologic regulators of SF production.

A protein activity that accumulates in rat serum after liver injury stimulates SF mRNA expression of SF mRNA in rat lung tissue and in MRC5 fibroblast cultures (Matsumoto et al., 1992*b*). The factor(s) responsible for this activity, called injurin, is a heat stable and trypsin-sensitive protein(s) with relative molecular mass of 10–30 kD, as determined by gel filtration chromatography and by elution of activity from an SDS–polyacrylamide gel. The source of injurin was not identified, but its appearance in the serum within several hours of organ injury suggests an endocrine mechanism for regulation of SF production. SF-IF has several properties in common with injurin, but a more definitive comparison is not possible since purification of injurin has not been reported.

Interestingly, various carcinoma cell lines that produce SF-inducing activity are unable to produce SF themselves (Table I). Since SF-IF is active at physiologic (picomolar) concentrations, it is likely that this factor acts through a specific cellular receptor. Thus, cells that produce SF-IF but not SF may lack the putative SF-IF receptor. Alternatively, these cells may be missing other components of the mechanism required for the expression of SF. In studies of cell types that produce SF, Stoker and colleagues (1987) observed that different lines of fibroblasts produced widely varying titers of SF. Even among strains of mouse 3T3 cells, the levels of SF production ranged from undetectable to very high. We can now postulate that differences in SF production rates result from differences in the expression of SF-IF and/or its receptor. This hypothesis is supported by the finding that three lines of non-SF–producing fibroblasts neither produced nor responded to SF-IF, while seven lines of SF-producing fibroblasts all produced detectable SF-IF activity.

In conclusion, we showed that SF production is regulated by autocrine and paracrine mechanisms involving a soluble low molecular weight protein that acts at picomolar concentrations. This scatter factor-inducing factor acts across species barriers and is biologically active on several stromal cell types. We suggest that SF-IF is an important physiologic regulator of SF production.

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