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.

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catter factor (SF) 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, 1990a; 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., 1990b, 1991, 1994). Although some epithelial cell lines produce modest...
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, 1990a; 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-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, W138, CCD32Lu, CLC13Lu, MRC5, CCL215, 3ASubE), mouse fibroblast (BALB/c-3T3, Swiss albino 3T3), human squamous carcinoma (A253, Calu), human glioblastoma (U87MG, Ha683), and human mammary carcinoma (SKBr3, MCF7, BT549, MDA231, 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 (Geriatric Radiation Cancer 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 (Bac2FS) cell lines were provided by Dr. B. Kacinski (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 macrophase 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 ras3T3 cells), 0.1 mM nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 µg/ml), as described earlier (Rosen et al., 1990a), 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., 1988). 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% CO2 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/cm²) 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 cm² 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/10⁶ 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, 1990a). 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

Immunol 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 Na2CO3 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 containing recombinant human SF (Genetch, 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 phosphate (50 µg/ml) (1 h at 37°C), and washed again. Color was developed by incubation with 100 µl of substrate solution (p-nitrophenyl phosphate [1 mg/ml], 1 M diethanolamine HCl, 2 mM MgCl2, 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/lanne) 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 washed again. Color was developed by incubation with 100 µl of substrate solution, and after autoradiography was performed. Densitometry was carried out using a Hoefer 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-1α and IL-1β) are a gift from Dr. R. Drummond (Cetus Corporation, Emeryville, CA). Recombinant murine IL-1α was a gift from Dr. P. Lucendo (Hoffman-LaRoche). 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, 1985). 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                                   |
| HuT38      | 6                                    |
| T47D       | 14                                   |
| 173T       | 11                                   |
| 186TL      | 13                                   |
| ras-3T3 (D4) | 24                               |

* Confluent tumor cells were incubated in DME (0.1 ml/cm²) for 48 h to produce CM. To assay the CM for SF-inducing activity, confluent MRC5 fibroblasts in 2-cm² 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%).

**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). Cells were grown to confluency in Falcon 1750-cm² 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-70 (Bio-Rad Laboratories, Cambridge, MA), a weak cation exchanger. 700 ml of filtrate was applied to a 1-kD membrane, and applied to a 10 ml bed (0.9 cm × 15 cm) Bio-Rex-70 column. This column was washed with Tris buffer and eluted with 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) Chromatography (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 NaHCO₃ buffer, pH 8. Eluates were dialyzed, dried, re-hydrated in 50 µl of DME, and assayed for SF-IF activity using MRC5 cells.
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-Seph- 
arose 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 di-
lution or by boiling for 5 min. Controls without pronase were treated iden-
tically.
‡ 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 di-
yalized using a 1-kD membrane, to remove DTT and iodoacetamide. Controls 
were incubated without DTT or iodoacetamide and dialyzed similarly.

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 bio-
assay 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 con-
ditions for collection of ras-3T3 CM for purification of SF (Rosen et al., 1990a), 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 chromatogra-
phy on S-Sepharose fast flow (Pharmacia Fine Chemicals), 
a resin with high affinity for SF (Rosen et al., 1990a). 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 in-
creases 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
After fractionation using an Amicon YM-30 membrane, properties of ras-3T3 SF-IF activity shows the densitometric ratios of SF (6 kb)/28S for control and 10% (v/v) of ras-3T3 CM 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. After fractionation using an Amicon YM-30 membrane, ras-3T3 SF-IF activity was found in the <30-kD filtrate. The concentrations of filtrate 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/cm²), 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/cm² 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/cm²), CCD11Lu (0.25 ml/cm²), and ras-3T3 (0.25 ml/cm²). 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 mRNAs 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 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 cultures treated without or with 10% (v/v) of ras-3T3 CM. Ras-3T3 SF-IF activity (BioRex-70 fraction) (Fig. 1 B). When assayed using the same collection volume (0.25 ml/cm²), 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/cm² 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/cm²), CCD11Lu (0.25 ml/cm²), and ras-3T3 (0.25 ml/cm²). 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.

**Sensitivity of Different Lines of Fibroblasts of SF-IF Activity**

Six of six human lung fibroblast lines (CCD11Lu, MRC5, W138, CCD32Lu, CCL33Lu, CCL215) were responsive to ras-3T3 CM <30-kD filtrate. The concentrations of filtrate 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/cm²), 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/cm² 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/cm²), CCD11Lu (0.25 ml/cm²), and ras-3T3 (0.25 ml/cm²). 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.

**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 <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.
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       | -             | -                | -              |
| SKB3                       | Human mammary carcinoma | -        | -                | -              |
| MCF7                       | "                  | -             | -                | -              |
| BT549                      | "                  | -             | -                | -              |
| MDA23                      | "                  | -             | -                | -              |
| MDA468                     | "                  | -             | -                | -              |
| Hz578T                     | "                  | -             | -                | -              |
| T47D                       | "                  | -             | -                | -              |
| I73T                       | "                  | -             | -                | -              |
| 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., 1992a; 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                  | MRC5 SF production rate (ng/ml) | U87MG SF production rate (ng/10^6 cells/24 h) | Hs683 SF production rate (ng/ml) | 1136TF SF production rate (ng/ml) |
|------------------------|---------------------------------|---------------------------------------------|---------------------------------|---------------------------------|
| 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-8 (20)              | 7.0                             | 1.0                                        | 4.5                             | 2.2                             |
| IL-11 (5)              | 5.6                             | NT                                         | NT                              | NT                              |
| TNFx (20)              | 13.7                            | 1.3                                        | 3.1                             | 2.2                             |
| Oncostatin-M (20)      | 6.9                             | 1.4                                        | 4.1                             | 3.4                             |
| Basic FGF (5)          | 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-cm² 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).

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

| Step                              | Activity (u) | Protein (μg) | Spec. Act. (u/μg) | Fold purification | Recovery (%) |
|-----------------------------------|--------------|--------------|-------------------|-------------------|--------------|
| 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         | 4,540             | 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.

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 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 NH₂-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.
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., 1990b, 1991; Weidner et al., 1990;...
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., 1992a; Tamura et al., 1993). We observed increases in SF protein and mRNA expression in multiple IL-1-treated cell lines, and combinations of IL-1 plus TNF did not stimulate SF production in several other stromal 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 x (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., 1992b). 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.

Supported in part by research grants from the United States Public Health Service (ROI CA50516) and the American Cancer Society (BE-7). Dr. Rosen is an Established Investigator of the American Heart Association.

Received for publication 19 April 1994 and in revised form 29 June 1994.

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