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Fibroblast Migratory Factor Derived from Mouse Colon Carcinoma Cells: Potential Roles of Fibronectin in Tumor Stroma Formation

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Abstract Mouse colon carcinoma cell line colon 38 was used to investigate migratory factor for fibroblasts because marked fibrotic tissue was associated with these cells growing at transplanted sites and liver metastases in vivo. Migration-inducing activity was detected in the serum-free culture supernatants of colon 38 cells, as shown by the Boyden chamber assays using NIH3T3 cells as target cells. The active substance was partially purified by a combination of anion-exchange, hydrophobic, and gel-permeation chromatography. An approximate relative molecular mass of the active substance was estimated to be between 100,000 and 400,000, judging from the eluting position in the gel-permeation chromatography. The migratory activity in the partially purified preparation was removed by incubation with beads coated with an anti-mouse fibronectin antibody. NIH3T3 cells incubated in the presence of culture supernatants of colon 38 cells exhibited higher growth rate, organized actin filaments, and increased chondroitin sulfate and hyaluronan. Fibronectin did not elicit such effects and partially purified migratory factor showed relatively low activity in these regards. Thus, colon 38 cells seem to secrete fibronectin and other soluble substances, which induce tumor stroma formation through migration and activation of host fibroblasts. J. Cell. Biochem. 80:635–646, 2001.

Key words: cell motility; colon 38; fibrosis; liver metastasis; protein purification

The generation of host-derived connective tissue, tumor stroma, is essential for the establishment of malignant tumor growth. Stromal components such as hyaluronan, collagens, and fibronectin regulate malignant behaviors through their influence on cell adhesion, migration, and gene expression. The predominant cells in tumor stroma are fibroblasts. Well-developed stromal tissue with a high content of type I collagen is often observed at the boundary of a malignant cell mass and surrounding host cells and termed as desmoplasia. The cause and biological consequence of tumor desmoplasia are not well defined. Growth and invasion of carcinoma are assumed to be dependent on fibrosis. Toole et al. [1979] reported that the hyaluronan content of invasive V2 carcinoma cells grown in rabbits was found to be 3–4 times greater than that of the same tumor grown in nude mice, where the tumor was noninvasive. Hyaluronan concentrations were highest at the interface between the tumor and the neighboring host tissue in the invasive rabbit tumors [Toole et al., 1979]. However, host stromal response and spontaneous lung metastasis were shown to have an inverse correlation [Barsky and Gopalakrishna,

Abbreviations used: BSA, bovine serum albumin; DMEM/F12, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium; DPBS, Dulbecco's phosphate buffered saline; FCS, fetal calf serum; mAb, monoclonal antibody; M_r, relative molecular mass; PAGE, polyacrylamide gel electrophoresis; ppCM, partial purified conditioned media; SDS, sodium dodecyl sulfate; SF-CM, serum-free conditioned media of colon 38 cells; TBS, Tris-buffered saline.

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1987; Hewitt et al., 1993; Nakanishi et al., 1994]. On the other hand, tumor-infiltrating myofibroblasts have been reported to play a role in the invasion and metastasis of colorectal carcinoma cells [Martin et al., 1996], and the extent of fibrosis indicated poor prognosis of colorectal carcinomas [Halvorsen and Seim, 1989; Ponz de Leon et al., 1992].

Fibroblasts are the predominant cells in tumor stroma. These cells are believed to be continuously recruited to tumor stroma from the surrounding tissue. They might also proliferate in situ. Because recruitment and proliferation of fibroblasts are also observed during wound healing, these processes are by no means unique to tumor tissue. Factors derived from inflammatory cells and platelets may play a central role in fibroblasts recruitment; however, it is also feasible that carcinoma cells can directly attract fibroblastic cell migration.

In the present study, we focused on the migration of fibroblastic cells toward the soluble products of colon 38 mouse colon carcinoma cells. These cells induce stroma formation when transplanted into a syngeneic host. We thought that a migratory factor released by colon 38 cells might be involved in the process. During our attempt to purify the migration-inducing substance to fibroblasts, we found that the activity is partly due to fibronectin produced by colon 38 cells. Although fibronectin has been shown to function as a chemoattractant for fibroblasts in vitro [Seppa et al., 1981], little attention was previously paid to the possibility that this glycoprotein plays a major role in carcinoma-induced stroma formation.

MATERIALS AND METHODS

Reagents

Azan-Mallory staining solution was from Sigma (St. Louis, MO). Anti-chondroitin sulfate monoclonal antibody (mAb) CS-56 was from Seikagaku Kogyo (Tokyo, Japan). Biotinylated goat anti-mouse IgG+A+M (H+L) and streptavidin-peroxidase were from Zymed (South San Francisco, CA). Biotin-labeled hyaluronan-binding protein and hyaluronidase (*Streptomyces hyalurolyticus*) were from Seikagaku Kogyo. Mouse fibronectin was purchased from Chemicon (Temecula, CA). Anti-laminin polyclonal antibody was from ICN-Cappel (Aurora, OH). Anti-fibronectin polyclonal antibody was from Biogenesis (Poole, UK).

Cell Lines and Cell Cultures

The mouse fibroblast cell line NIH3T3 was obtained from the American Type Culture Collection (ATCC). Mouse colon carcinoma cell line colon 38 [Corbett et al., 1975] was kindly provided by Dr. Takashi Tsuruo of the Institute of Molecular and Cell Biology, The University of Tokyo, Japan. The mouse embryonic fibroblast cell line BALB/C CL.7 was from ATCC. The mouse liver fibroblast cell line was from Dr. Takao Yamori of the Japanese Foundation for Cancer Research, Cancer Chemotherapy Center, Tokyo, Japan. Mouse skin primary fibroblasts were also used. All cell lines were cultured in an 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12: Nissui, Tokyo, Japan) containing 10% heat-inactivated fetal calf serum (FCS: Intergen Co., Purchase, NY) in a humidified atmosphere with 95% air, 5% CO_2 at 37°C.

Immunohistochemical Examination of Tumors Derived from Colon 38 Cells

Colon 38 cells, 1.75×10^6 cells in 50 μl, suspended in 25 mM HEPES-DMEM/F12 (FCS-free) at 3.5×10^7 cells/ml, were intrasplenically injected into female 5-week-old C57BL/6 mice (SLC Japan, Shizuoka, Japan). Three weeks later, the mice were anesthetized with ether and the primary tumor and liver metastases were surgically removed. The spleens and livers were fixed with acetone, methyl benzoate, and xylene and embedded in paraffin. Sections (4 μm) were stained with hematoxylin-eosin or by the Azan-Mallory method. Sections were also blocked with 2% normal goat serum and 3% bovine serum albumin (BSA) in Dulbecco's phosphate buffered saline (DPBS) and processed for immunohistochemical stainings.

The sections were incubated with monoclonal anti-chondroitin-sulfate antibody CS-56 (Seikagaku Kogyo; diluted 1/200 in 2% normal goat serum, 3% BSA in DPBS) at room temperature for 40 min. Biotinylated-goat anti-mouse IgG+A+M (H+L) was used at 1/200 dilution in 2% normal goat serum and 3% BSA in DPBS at room temperature for 30 min. The binding of mAb was histochemically detected using Histomark Red (Kirkegaard and Perry, Gaithersburg, MD), and the cell nucleus was counterstained in Mayer's hematoxylin. The sections were observed under a microscope and photographed (Olympus, Tokyo, Japan).

Normal mouse serum was used in place of the first antibody as a negative control.

To histochemically detect hyaluronan, the sections were treated with 0.1% trypsin in 0.05 M Tris–HCl (pH 7.4) at 37°C for 5 min, blocked with 3% BSA in PBS and reacted with 5 μg/ml biotinylated hyaluronan binding protein (Seikagaku Kogyo). Alkaline phosphatase-labeled streptavidin (Zymed; 1/500 dilution in 20 mM Tris–HCl (pH 7.4), 0.15M NaCl) and Histomark Red were used. The nucleus was counterstained with Mayer's hematoxylin. As a negative control, the sections were pretreated with 200 turbidity-reducing Units/ml of hyaluronidase in 100 mM sodium acetate buffer, pH 6.0, at 60°C for 2 h and stained [Asari et al., 1992].

Preparation of Serum-Free Conditioned Media of Colon 38 Cells (SF-CM)

SF-CM was prepared as follows. Briefly, 1.8×10^5 cells were seeded into 100-mm plastic dishes with 12 ml of DMEM/F12 containing 10% FCS and incubated at 37°C for 24 h. The cells were washed twice with PBS and incubated for 2 days with 12 ml of ASF104 medium (Ajinomoto, Tokyo, Japan). The obtained supernatants were centrifuged at 1,000*g* for 20 min to remove debris and then passed through nitrocellulose filters (pore size 0.45 μm; Iwaki, Chiba, Japan) and stored at 4°C until use.

Partial Purification of Migration-Inducing Substances from SF-CM

Solid ammonium sulfate was added to SF-CM (6470 ml) to adjust to 60% saturation. The precipitate collected by centrifugation was dissolved in water and dialyzed to 10 mM phosphate buffer (pH 8.0), and loaded onto a Mono Q anion exchange column (0.5×5 cm) (Pharmacia, Uppsala, Sweden). The column was eluted with 10 mM phosphate buffer (pH 8.0) until absorbance at 280 nm reached the baseline. The column was eluted with 50 ml of a linear gradient of 0 to 0.6 M NaCl. The fractions eluted from 0.35 to 0.49 M NaCl were combined and used in the next step. All fractions were concentrated using Centriprep (30,000 molecular weight cut off) (Amicon, Beverly, MA), and dialyzed to 20 mM Tris–HCl buffer (pH 7.4). The sample solution was made to 0.6 M $(NH_4)_2SO_4$ and loaded onto a phenyl-Superose (0.5×5 cm) (Pharmacia). The column was eluted first with 0.6 M $(NH_4)_2SO_4$ in 20 mM Tris–HCl (pH 7.4), until absorbance at 280 nm reached the baseline. The column was then eluted with 20 ml of a linear gradient of 0.6 to 0 M $(NH_4)_2SO_4$. Fractions eluted with 0 M $(NH_4)_2SO_4$ contained migration-inducing activity were combined for the next step. They were concentrated using Centricon (30,000 molecular weight cut off) (Amicon). The samples were loaded onto a Superose 6 (1.0×30 cm) (Pharmacia) and eluted with PBS. Fractions were monitored for their migration-inducing activity for NIH3T3 cells by Boyden chamber assays.

Immunoprecipitation

Protein A-Sepharose CL-4B (Pharmacia) was incubated with preimmune serum or polyclonal antibody to mouse fibronectin (Biogenesis) or polyclonal antibody to mouse laminin (ICN-Cappel) for 1 h at 4°C. The beads were rinsed by centrifugation and incubated with partial purified conditioned media (ppCM) for 3 h at 4°C. Supernatants were recovered by centrifugation. Migration-inducing activity of the supernatants was assessed by Boyden chamber assays.

Polyacrylamide Gel Electrophoresis and Western Blotting Analysis

ppCM of untreated or treated with rabbit preimmune serum or anti-fibronectin antibody were treated with 67.25 mM Tris–HCl buffer (pH 6.8) containing 1% sodium dodecyl sulfate (SDS), 2.5% 2-mercaptoethanol, 5% glycerin, and 1 mM EDTA at 100°C for 5 min. These samples were separated by polyacrylamide gel electrophoresis (PAGE) on 5% gels in the presence of 0.1% SDS, according to the method of Laemmli (SDS-PAGE). To visualize protein components, silver staining by the use of 2D-Silver Stain Agent (Daiichi Pure Chemicals, Tokyo, Japan) was performed. The samples were also electroblotted onto a polyvinylidene difluoride membrane (Immobilon-Psq Transfer Membrane, Millipore, Bedford, MA) using a Milli Blot-SDE system (Millipore) in Western blotting analysis. Membranes were soaked in DPBS containing 3% BSA at 4°C overnight, followed by incubation with anti-fibronectin antibody diluted at 1:400 in 10 mM Tris-buffered saline (TBS) containing 0.05% Tween 20 and 3% BSA at room temperature for 1.5 h with gentle shaking. After being washed five times with TBS containing 0.05% Tween 20,

the membranes were incubated with alkalinephosphatase-conjugated goat anti-rabbit IgG(H+L) (Zymed) diluted at 1:1000 in TBS containing 0.05% Tween 20 at room temperature for 50 min. Bound antibodies were visualized with Alkalinephosphatase Substrate Kit II (Vector, Burlingame, CA).

Boyden Chamber Assays

Migration-inducing activity to NIH3T3 cells was determined by Boyden chambers with polycarbonate filters (8 μm pore diameter; Kurabo, Osaka, Japan). Lower chambers (750 μl) contained substances to be assayed. The upper chamber contained 1×10^5 fibroblasts and 350 μl of ASF104 media. After incubation at 37°C for 24 h, the chambers were removed and the number of cells in the lower chambers was counted. These assays were performed in triplicate in a multi-blind well apparatus. In some experiments, antibodies or synthetic peptides (cyclo (GRGDSPA) peptide or cyclo $(RSarDPhg)_2$ peptide provided by Dr. Hiromichi Kumagai of Asahi Glass, Yokohama, Japan) was added to the lower chambers.

Cell Growth In Vitro

NIH3T3 cells (1×10^4) were seeded on a 96-well plastic plate and incubated with ASF104 medium for 4 h. The medium in the wells was replaced with ASF104 containing ppCM or mouse fibronectin. After 72 h, the cells were fixed with 0.25% glutaraldehyde in PBS and stained with 0.2% crystal violet dissolved in 20% ethanol in PBS. After the addition of 10% methanol/40% ethanol, the absorption was determined at 550 nm with a Bio-Rad Microplate Reader (Bio-Rad, Hercules, CA) and used as an indicator of the number of cells.

Staining of Actin Filaments

NIH3T3 cells were trypsinized, suspended in ASF104 medium, and seeded on chamber slides. Two hours later, ASF104 medium was replaced with test solutions and incubated for 24 h. The cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) at room temperature for 20 min and treated with 0.1% Triton X-100 in PBS at room temperature for 30 min. After being rinsed in 0.1 M glycine in PBS for 5 min, the cells were stained with tetramethylrhodamine isothiocyanate-conjugated phalloidin (Sigma, St. Louis, MO) diluted in DPBS (4.6×10^{-11} M) at room temperature for 20 min. The slides were rinsed in DPBS and mounted in Aqua Poly Mount (Polysciences, Warrington, PA). The cells were examined under an epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a laser confocal system (Bio-Rad).

Biosynthesis of Chondroitin Sulfate and Hyaluronan

NIH3T3 cells (2×10^4) were seeded on a 96-well plastic plate and incubated with ASF104 medium for 4 h. The medium in the wells was replaced with solutions to be tested. At given time points, the cells were fixed with 0.1% glutaraldehyde in DPBS, rinsed, and reacted with anti-chondroitin sulfate mAb CS-56 or biotinylated hyaluronan-binding protein. The binding of mAb CS-56 was measured by the addition of biotinylated goat anti-mouse IgG. Streptavidin-peroxidase and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt solution were used.

RESULTS

Histological Examination of Tumors Derived from Colon 38 Cells

Sections of liver metastasis derived from intrasplenically injected colon 38 cells stained with hematoxylin and eosin, Azan staining, anti-chondroitin sulfate antibody (mAb CS-56), or biotinylated hyaluronan-binding protein are shown in Figure 1. Extracellular matrices and fibroblastic cells were observed in the tumor tissue (Fig. 1). The area where clusters of tumor cells were seen was also noted by strong staining with the Azan staining method, indicating collagen deposits. The same area was positive for anti-chondroitin sulfate mAb and hyaluronan-binding protein. Because chondroitin sulfate was not produced by colon 38 cells growing in vitro, its presence seemed to be attributable to infiltrating host cells. Thus, we hypothesized that fibroblasts migrate toward substances secreted by colon 38 cells.

Detection of Migration-Inducing Activity in Culture Supernatants of Colon 38 Cells

We tested serum-free culture supernatants of colon 38 cells (SF-CM) for migration-inducing activity directed to fibroblasts. NIH3T3 cells, BALB/C CL.7 cells, mouse liver fibroblasts, and mouse skin primary fibroblasts were used. The results of Boyden chamber assays

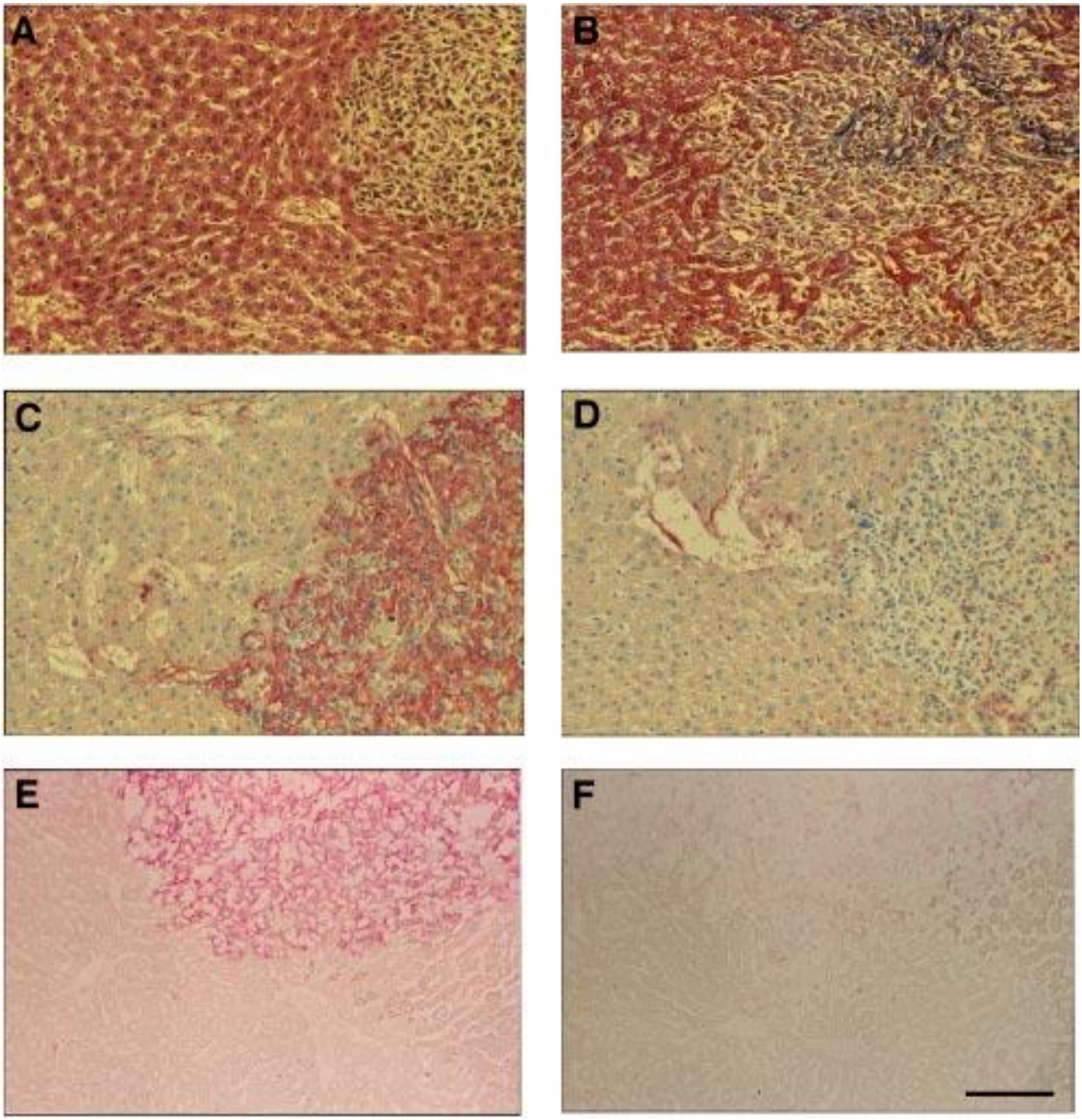

Fig. 1. Histological appearance of experimental liver metastasis of colon 38 cells. (**A**) Hematoxylin and eosin staining, (**B**) Azan staining, (**C**) staining with anti-chondroitin sulfate mAb CS-56, (**D**) negative control for (C) with mouse IgG instead of mAb CS-56, (**E**) staining with biotinylated hyaluronan-binding protein, (**F**) negative control for (E) after treatment of sections with hyaluronidase. Extracellular matrices and fibroblast-like cells were observed within the tumor tissue. All panels are microscopic photographs under the same magnifications. A bar in panel (F) indicates 100 μm.

suggested that the culture supernatants contained a chemoattractant. Because these fibroblasts migrated to the lower chambers even when SF-CM was added to both upper and lower chambers, SF-CM seemed to contain a factor which promoted random motility of these cells, too. Thus, the factor was tentatively termed migratory factor. The migration-indu-

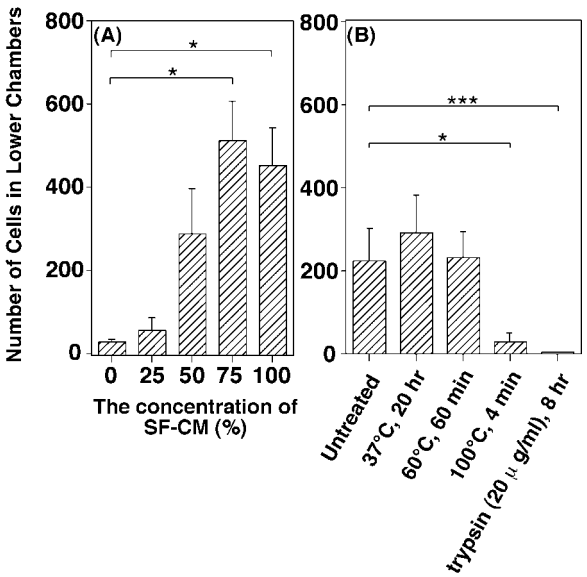

Fig. 2. Preliminary characterization of a migration-inducing activity toward fibroblasts. (**A**) Dose-dependency of the migration-inducing activity of SF-CM, (**B**) Migration-inducing activity of SF-CM after treatments at different temperatures or with trypsin. SF-CM was heated at 37°C for 20 h, at 60°C for 60 min, or at 100°C for 4 min. The treatment with trypsin (20 μg/ml) was performed at 37°C for 8 h. The migration-inducing activity was destroyed by treatment with trypsin and significantly decreased after heating at 100°C for 4 min. Bars represent standard deviation from triplicate experiments. Statistical significance of the differences are indicated by asterisks ($^{*}P < 0.05$, $^{**}P < 0.001$).

cing effect was most prominent when NIH3T3 cells were used and the results with these cells are shown in Figure 2. The activity was dependent on the concentration and destroyed by heating or by treatment with trypsin (20 μg/ml) for 8 h. The culture supernatants of NIH3T3 cells did not seem to contain an autocrine motility factor.

Partial Purification of Migratory Factor from Culture Supernatants of Colon 38 Cells

We attempted to optimize the sequential chromatography conditions in order to purify the migratory factor. By using Mono Q, phenyl-superose, and Superose 6, the migration-inducing substance was purified 33-fold when the specific activity was calculated based on the dilution of the fractions to result in the migration of a similar number of cells to the lower chambers in the motility assays. At each step of chromatographic purifications, migration-inducing activity was monitored after dialysis against PBS. The results showed that there was a predominant peak of activity at each step. The components corresponding to migration-inducing activity were pooled and processed to the next step. After these procedures, ppCM was obtained. Details of the purification are summarized in Table 1. When 6470 ml of SF-CM was used, 2.5 ml of ppCM (0.064 mg protein/ml) was obtained. This preparation usually contained migratory factor that induced the migration of NIH3T3 cells to a lower chamber within 24 h, at 1:24 dilution. We defined 1 unit of migration-inducing activity that induced migration of 100 cells to a lower chamber in 24 h.

From the eluting position on Superose 6, the molecular mass of the active protein was estimated to be between 100,000 and 400,000. At this step, the fractions with migration-inducing activity showed several protein bands when separated by gel electrophoresis in the presence of sodium dodecylsulfate under reducing conditions and stained by the silver staining method. The electrophoretic profile was reproducible from one experiment to another as shown in lane 1 in Figure 3. The major components having approximate relative molecular mass (M_r) 210,000 and 185,000 were reactive with anti-fibronectin antibodies (Fig. 3). Components with lower apparent M_r were not reactive with anti-fibronectin mAb.

A Possibility That This Migratory Factor is Fibronectin

Because of the high M_r, the migratory factor might be an extracellular matrix component. When ppCM was incubated with beads coated with anti-fibronectin antibody, the activity dropped to approximately 10% (Fig. 4). Anti-laminin antibody did not have such an effects. The untreated or anti-fibronectin-treated ppCM were separated by gel electrophoresis in the presence of sodium dodecylsulfate under reducing conditions and tested for the binding of anti-fibronectin antibody. As shown in Figure 3, two major bands with approximate M_r 210,000 and 185,000 were abolished by the treatment. Furthermore, 10^{-6} M of cyclo (GRGDSPA) peptide completely suppressed the migration-inducing activity of ppCM (Fig. 5). The control peptide did not show such activity. We also observed that mouse plasma fibronectin at a concentration of 0.5 μg/ml elicited very similar migration-inducing activity directed to NIH3T3 cells as ppCM (containing 0.5 μg/ml

TABLE I. Purification of ppCM from SF-CM

Fractions	Volume (ml)	Total protein[a] (mg)	Chemotactic activity[b] (U)	Specific activity (Units/mg)	Fold
SF-CM	6470	352.6	6470	18.5	1
$(NH_4)_2SO_4$ precipitate	139.9	49.0	1117	22.8	1.2
Mono Q pool fractions	13.5	7.14	871.1	122.0	6.6
Phenyl-superose pool	4.0	0.88	123.3	140.1	7.6
ppCM	25	0.16	97.68	520.3	33.0

[a]Protein was quantitated by the Bio-Rad protein assay using BSA as standard.
[b]One unit was defined as chemotactic activity that causes migration of 100 cells to the lower chamber in 24 h.

fibronectin). Therefore, the migratory factor observed in the SF-CM seemed to be accounted for fragments of fibronectin, although we were not able to determine the N-terminal sequence

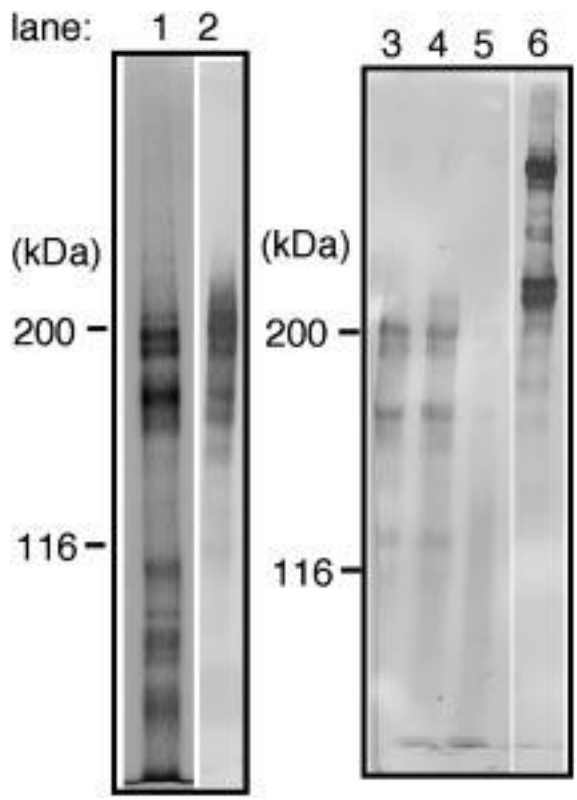

Fig. 3. Electrophoretic profiles of ppCM revealed by silver staining (**lane 1**) and Western blotting with anti-fibronectin antibodies (**lanes 2–6**). ppCM were separated by polyacrylamide gel electrophoresis in the presence of SDS on 5% gels, electroblotted onto polyvinylidene difluoride membranes. Lane 1, untreated ppCM (0.5 μg protein); lane 2, untreated ppCM (1.0 μg protein); lane 3, ppCM (5 μg protein) treated with uncoated beads; lane 4, ppCM (5 μg protein) treated with beads coated with rabbit preimmune serum; lane 5, ppCM (5 μg protein) treated with beads coated with rabbit anti-fibronectin antibody, and lane 6, purified mouse fibronectin (1.0 μg protein).

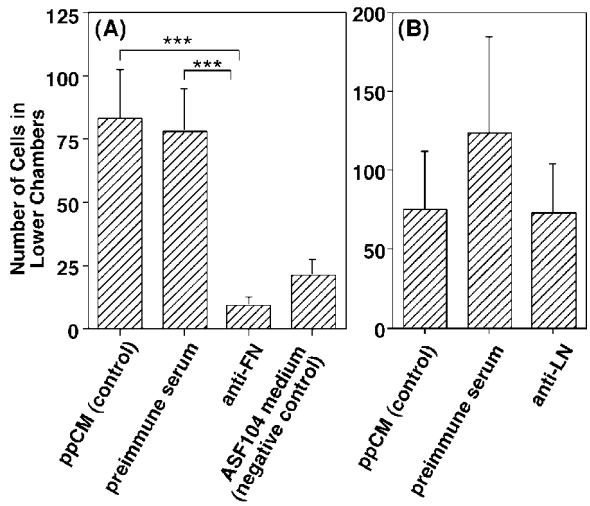

Fig. 4. Motility of NIH3T3 cells after treatment of ppCM with anti-mouse fibronectin (FN) (**A**) or anti-mouse laminin, (**B**) antibodies, as determined by Boyden chamber assays. Preimmune serum, anti-fibronectin mAb, or anti-laminin mAb was incubated with Protein A-Sepharose CL-4B for 1 h at 4°C. The beads were retrieved by centrifugation and rinsed with DPBS. Then, ppCM (containing 0.5 μg/ml fibronectin) was incubated with antibody-coated protein A-Sepharose CL-4B for 3 h at 4°C. Supernatants were tested for migration-inducing activity by Boyden chamber assays. Bars represent standard deviation from triplicate experiments. Statistical significance of the differences are indicated by asterisks ($P < 0.001$).

of these two protein components retrieved from the gels. It has been reported that fragments of cellular fibronectin having M_r 210,000 and 185,000 are generated by the action of serine proteases [Sekiguchi et al., 1985].

Influence of ppCM and Fibronectin on Fibroblasts

We examined effects of ppCM and fibronectin on the activation of NIH3T3 cells other than migration. ppCM stimulated fibroblast proliferation in a concentration-dependent manner,

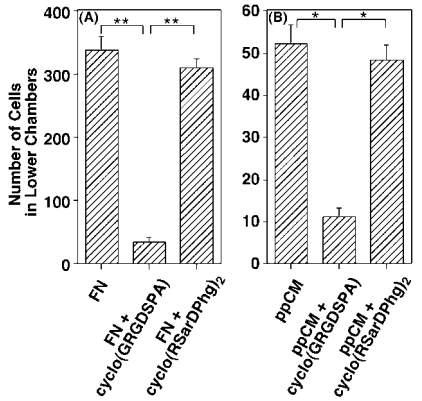

Fig. 5. Inhibition of motility of NIH3T3 cells by cyclo(GRGDSPA) in Boyden chamber assays. Fibronectin (FN; 0.5 μg/ml, 0.75 ml /well) (**A**) or ppCM (corresponding to 0.5 μg/ml fibronectin) (**B**) was mixed with cyclo (GRGDSPA) or cyclo $(RSarDPhg)_2$ and added to the lower compartment. NIH3T3 cells (1×10^5) were added to the upper compartment. After incubation at 37°C for 24 h, cells that had migrated to the lower chambers were counted. Bars represent standard deviations from triplicate experiments. Statistical significance of the differences are indicated by asterisks ($^{**}P < 0.01$, $^{*}P < 0.025$).

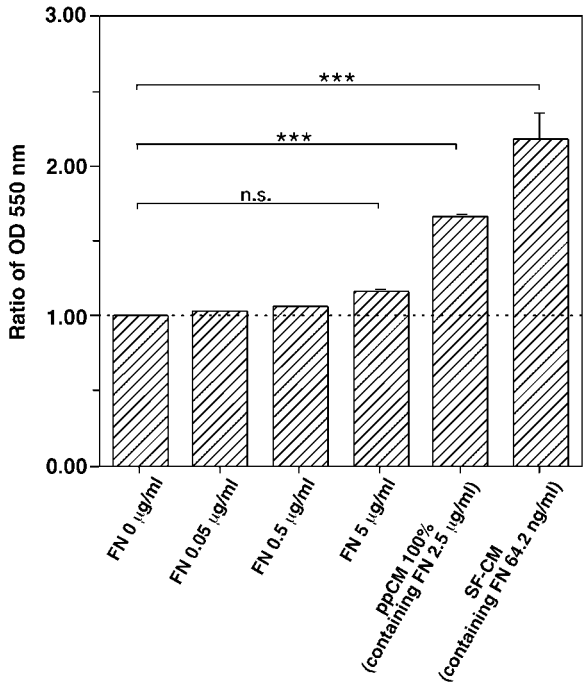

Fig. 6. The effect of SF-CM, ppCM or fibronectin on the growth of NIH3T3 cells. NIH3T3 cells (1×10^4) were seeded on a 96-well plastic plate and incubated with ASF104 medium for 4 h. Media in the wells were replaced with ASF104 medium containing mouse fibronectin or ppCM. The amount of fibronectin in SF-CM and ppCM was determined by inhibition of anti-fibronectin mAb and indicated in the figure. After 72 h, the cells were fixed with 0.25% glutaraldehyde in PBS and stained with 0.2% crystal violet dissolved in 20% ethanol in PBS. The dye was solubilized by the addition of 10% methanol/40% ethanol in water, and the absorbance at 550 nm was determined on a Bio-Rad Microplate Reader. The value is presented as a ratio to the absorbance of control cultures. Bars represent standard deviations from triplicate experiments. Statistical significance of the differences are indicated by asterisks ($P < 0.005$). n.s., non significance.

while fibronectin did not stimulate fibroblast proliferation (Fig. 6). We also tested the influence of ppCM and fibronectin on the organization of actin filaments in NIH3T3 cells. Cells treated with ppCM or fibronectin for 24 h were fixed and stained with tetramethylrhodamine isothiocyanate-labeled phalloidin. Actin fibers of these cells were shown to be highly organized after treatment with ppCM, but fibronectin at 0.5 μg/ml failed to induce reorganization of actin (Fig. 7). Thus, reorganization of actin does not seem to have correlation with the cellular motility in this experimental system. Cell surface expression of chondroitin sulfate was enhanced about 1.4-fold by ppCM containing 1.25 μg/ml fibronectin. Hyaluronan levels doubled as a result of the same concentration of ppCM. In the presence of fibronectin (0.5 μg/ml), expression of chondroitin sulfate was about 1.2-fold, which was higher than in the control, whereas hyaluronan on cell surfaces of NIH3T3 cells did not change (Fig. 8). Thus, fibronectin does not seem to contribute to these responses of fibroblasts to ppCM.

We concluded that colon 38 cells produce and secrete multiple substances that can induce migration and stimulation of fibroblasts. Fragments of fibronectin appeared to be predominant component representing this activity.

DISCUSSION

When mouse colon carcinoma colon 38 cells were transplanted into syngeneic C57BL/6 mice, marked fibrotic responses were observed at the sites of inoculation (spleen) and in liver metastases, as shown by the immunohistochemical detection of collagen, chondroitin sulfate, and hyaluronan. A similar morphological feature was seen when the cells were orthotopically transplanted. Therefore, we hypothesize that products of colon 38 cells directly influence fibroblast motility and gene expression. SF-CM was shown to contain a migratory factor to mouse fibroblast NIH3T3 cells, as measured by Boyden chamber assays,

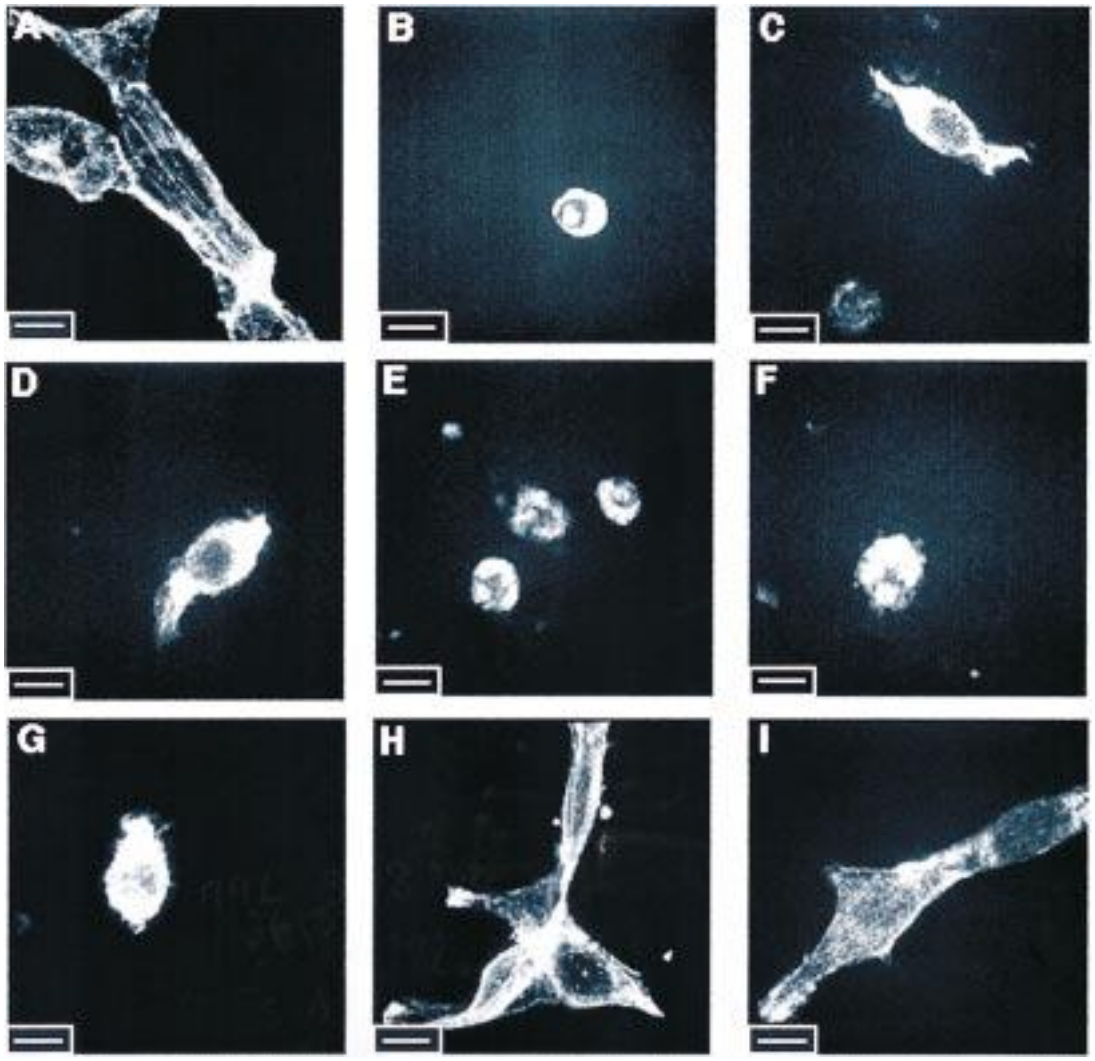

Fig. 7. The effect of fibronectin or ppCM on the actin organization of NIH3T3 cells. NIH3T3 cells were trypsinized and suspended in ASF104 medium and plated into chamber slides. The medium was replaced with ASF104 medium containing fibronectin or ppCM 2 h later and then further incubated for 24 h. The cells were fixed, permeabilized, and incubated with phalloidin, as described in the Materials and Methods section. Cells were incubated in: (**A**) an 1:1 mixture of DMEM and Ham's F12 media FCS 10%, (**B**) ASF104 media, (**C**) fibronectin 0.05 μg/ml, (**D**, **E**) 0.5 μg/ml of fibronectin, (**F**) 5 μg/ml of fibronectin, (**G**) 12.5% ppCM containing 0.3125 μg/ml fibronectin, (**H**) 25% ppCM containing 0.625 μg/ml fibronectin, and (**I**) 50% ppCM containing 1.25 μg/ml fibronectin. Bars represent 10 μm.

and to stimulate the production of extracellular matrix components in vitro. SF-CM also seemed to stimulate random motility of these cells.

We attempted to purify the migration-inducing substance that was likely to be a protein. Ammonium sulfate precipitation, Mono Q anion exchange chromatography, phenyl-superose hydrophobic interaction chromatography, and Superose 6 gel permeation chromatography were applied. At each step of purification by a combination of different types of column chromatography, the active material eluted as a major single peak. The molecular weight of fibroblast migratory factor was estimated to be 100,000–400,000, as indicated by the elution position on Superose 6. By using sodium dodecylsulfate-polyacrylamide gel electrophoresis under reducing conditions, the pooled partially purified migratory factor fractions after Superose 6 (ppCM) were shown to contain several protein components detectable after silver staining.

Both ppCM and mouse plasma fibronectin induced fibroblast migration. These effect of ppCM was completely abolished when ppCM was treated with anti-fibronectin-coated beads.

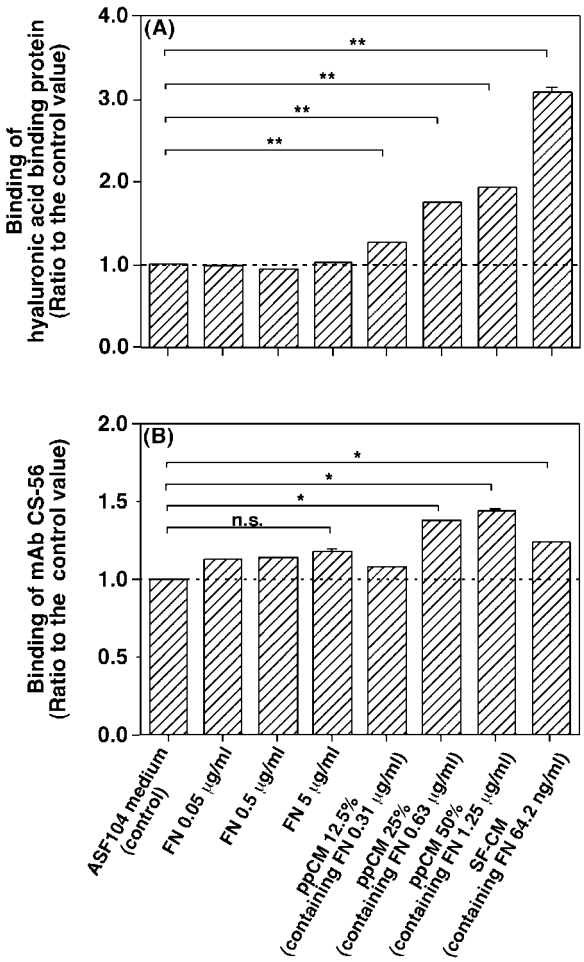

Fig. 8. The influence of SF-CM, ppCM or purified mouse fibronectin on the production of hyaluronan (**A**) and chondroitin sulfate, (**B**) by NIH3T3 cells as shown by enzyme-linked immunosorbent assays. FN, fibronectin. Bars represent standard deviations from triplicate experiments. Statistical significance of the differences are indicated by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$). n.s., non-significance.

After this treatment components having approximate M_r 210,000 and 185,000 were eliminated. These components were approximately corresponding to tryptic fragments of cellular fibronectin [Sekiguchi et al., 1985]. Proliferation of fibroblasts in vitro was induced by ppCM but not by fibronectin. These results strongly suggested that fragments of fibronectin produced and released by colon 38 cells are responsible for the ppCM-induced migration of fibroblastic cells. Reorganization of actin filaments and production of extracellular matrix components induced by ppCM was not accounted by fibronectin.

Fibronectin is a well-characterized glycoprotein mediating cell-to-extracellular matrix interactions. A number of investigations have demonstrated that this glycoprotein elicits multiple biological activities that potentially influence tumor growth and metastasis in vivo [Mosher, 1980; Hynes and Yamada, 1982]. Fibronectin is known to be chemotactic for endothelial cells [Bowersox and Sorgente, 1982], and it may play an important role in vascular stroma formation [George et al., 1997]. Fibronectin is also known to modulate endothelial response to growth factors [Madri et al., 1988] and to promote the elongation of microvessels during angiogenesis in vitro [Nicosia et al., 1993]. Also, tumor-associated fibroblasts are known to produce fibronectin fragments that increase migration of tumor cells [Hu et al., 1995, 1997].

Chemotaxis of fibroblasts toward fibronectin was studied in detail by Seppa and co-workers [Seppa et al., 1981]. They showed that a 160-kDa peptide fragment of fibronectin that contained heparin-binding and cell-binding domains had chemotactic activity, while a 40-kDa peptide containing a collagen-binding domain was inactive [Seppa et al., 1981]. Postlethwaite suggested that fibronectin and fragments of fibronectin function in vivo as specific chemoattractants for fibroblasts [Postlethwaite et al., 1981]. Directional migration of fibroblasts may be induced by these substances to sites of tissue injury, remodeling, morphogenesis, or malignant tumors. A domain responsible for the migration of fibroblasts was identified between the collagen-binding site and the major heparin-binding site (about 170 kDa apart from the N-terminal and about 70 kDa from the C-terminal ends of the two subunit peptide chains) [Albini et al., 1983]. Rat plasma contains at least two chemotactically active fragments of fibronectin, components that cooperatively regulate the chemotactic migration of mouse embryo fibroblastic cells [Fukai et al., 1991].

Our results are consistent with these previous reports showing that fragments of fibronectin elicit strong migration-inducing activity towards fibroblasts. Schor proposed that the gelatin-binding domain played a role in the control of cell migration during pathological processes, such as tumor invasion and wound repair [Schor et al., 1996]. Judging from the eluting position on a Superose 6 column, fibronectin released into the culture-conditioned media of colon 38 cells seemed to have a size

close to that of the tryptic fragments (210,000 and 185,000). There was a possibility that these fragments have additional activity which was not elicited by native fibronectin. However, the activity promoting the growth of fibroblast detected in SF-CM is not likely to be associated with fragmented fibronectin. The majority of the growth factor activity eluted at a different position in the Superose 6 chromatography. Thus, similar activity in ppCM seemed to be due to small quantity of contaminating growth promoting proteins.

Many growth factors are known to influence fibroblast motility. These include transforming growth factor-β [Postlethwaite et al., 1987], platelet-derived growth factor [Seppa et al., 1982], interleukin-8 [Dunlevy and Couchman, 1995], basic-fibroblast growth factor [Rifkin and Moscatelli, 1989], epidermal growth factor [Adelmann-Grill et al., 1989], interleukin-4 [Postlethwaite and Seyer, 1991], tumor necrosis factor-α [Postlethwaite and Seyer, 1990], and migration stimulating factor [Schor et al., 1988a, 1988b]. The possibility that some of these substances, potentially produced by colon 38 cells, influence motility and other phenotypes of fibroblasts cannot be eliminated because their diverse effects are often exerted depending on the concentration. It remains to be elucidated whether fibronectin is the major contributor of colon 38 cell-induced tumor stroma formation. Clonal subpopulations of these cells without strong inducibility of tumor stroma in vivo in syngeneic mice will prove or disprove such possibilities.

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