Induction of Hepatocyte Growth Factor/Scatter Factor by Fibroblast Clustering Directly Promotes Tumor Cell Invasiveness

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Abstract

For determining the malignant behavior of a tumor, paracrine interactions between stromal and cancer cells are crucial. We previously reported that fibroblast clustering induces cyclooxygenase-2 (COX-2), plasminogen activation, and programmed necrosis, all of which were significantly reduced by nonsteroidal anti-inflammatory drugs (NSAID). We have now found that tumor cell–conditioned medium induces similar fibroblast clustering. Activation of the necrotic pathway in clustering fibroblasts, compared with control monolayer cultures, induced a massive >200-fold production of bioactive hepatocyte growth factor/scatter factor (HGF/SF), which made human carcinoma cells spread and invade a collagen lattice. This response occurred only if a functional, properly processed c-Met receptor was present, which was then rapidly phosphorylated. The invasion-promoting activity was inhibited by a neutralizing HGF/SF antibody. NSAIDs, if added early during fibroblast aggregation, inhibited HGF/SF production effectively but had no effect at later stages of cell aggregation. Our results thus provide the first evidence that aggravated progression of tumors with necrotic foci may involve paracrine reciprocal signaling leading to stromal activation by direct cell-cell contact (i.e., nemosis). (Cancer Res 2005; 65(21): 9914-22)

Introduction

Several types of human solid tumors are histologically complex mixtures composed not only of tumor cells but also of normal nonneoplastic cells such as fibroblasts, endothelial, smooth muscle, and immune cells (1, 2). Fibroblasts usually markedly outnumber the other cell types and may in some circumstances constitute over 90% of tumor mass (3). Because of their high numbers within the tumor mass, stromal fibroblasts are prime targets for tumor-derived paracrine mediators such as growth factors and cytokines. We and others have shown that tumor cells such as melanoma cells produce soluble mitogenic factors for fibroblasts (4, 5) that may contribute to fibroblast hyperplasia and reactive stroma within and around growing tumors (6, 7). Stromal contributions have been strongly linked to cancer progression, metastasis, and growth (ref. 8; e.g., in cutaneous melanoma; ref. 9).

Mesenchymal or stromal fibroblasts are the primary source of the hepatocyte growth factor/scatter factor (HGF/SF) promoting cell motility, morphogenesis, and proliferation under normal and pathophysiologic conditions including cancer (10–12). These effects of HGF/SF are mediated through a specific tyrosine kinase receptor, c-Met (13, 14). Expression of the *c-met* proto-oncogene first results in a 190-kDa precursor protein, from which the mature receptor, composed of a 50-kDa α-subunit and a 145-kDa β-subunit linked by disulfide bonds, is then cleaved (15). Overexpression of c-Met and increased production of HGF/SF in tumor tissues has been related to aggressive tumor growth as well as to poor therapeutic outcome (16, 17). Research has mainly focused on c-Met signaling and its differing mutations and expression in cancer cells (13, 18, 19), with HGF/SF production receiving less attention. The preeminent role of fibroblast HGF/SF as a key regulator of tumor cell morphogenesis and increased invasiveness is, however, appreciated (11, 20), outlining its importance in paracrine signaling within the tumor microenvironment. Mechanisms leading to induction and functional activation of HGF/SF in tumor-associated fibroblasts have nevertheless remained largely obscure.

Multicellular spheroids of tumor cells frequently serve as a model system for nodular growth *in vitro* (21). These three-dimensional culture systems mimic certain control mechanisms operating *in vivo* under several pathophysiologic conditions (22). Nodular growth is common for malignant, fast-growing tumors but can also occur in the skin as hyperproliferation of dermal fibroblasts characterized by distinctly greater cell density, as in dermal fibrosarcomas (23). Normal adherent cells, however, do not thrive when depleted of matrix contacts, and also without cell-cell contacts, they undergo anoikis (24). We recently discovered that an endogenous nonapoptotic death program is triggered within dermal fibroblasts by direct homotypic cell-cell interactions, and that this type of programmed cell death is associated with excessive production of inflammation-associated prostaglandins (25). This pathway of cell death showed typical morphologic features of necrosis by electron microscopy, with no activation of molecules related to apoptosis (25).

Our results thus provided the first molecular insight into a novel type of fibroblast reactivity. As the presence of necrotic foci within tumors is linked to aggressive cancer progression and unfavorable prognosis (26, 27), we sought to show here that parallel paracrine signaling between tumor cells and fibroblasts can result in formation of fibroblast clustering by direct cell-cell contacts and that such fibroblast activation can in turn contribute to increased tumor growth and invasiveness. These studies provide a molecular link and rationale between necrosis and enhanced tumor aggressiveness. We showed that nonsteroidal anti-inflammatory drugs (NSAID) inhibited cell-cell contact–induced fibroblast activation and necrotic death (25). As long-term use of NSAIDs shows chemotherapeutic efficacy and reduced risk for melanoma as well as for cancers of the breast, prostate, and lung in several epidemiologic, clinical, and experimental studies (28, 29), we examined NSAID effects on

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cell-cell contact–induced production of HGF/SF by activated fibroblasts. In addition, we aimed to show how time dependent was the effect of NSAIDs in respect to progression of cell-cell contact formation.

Materials and Methods

Cell lines. Human melanoma cell lines WM-75 and WM-373 were kindly provided by Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA); the Bowes melanoma cell line came from the American Type Culture Collection (Rockville, MD); human carcinoma cell lines TE-1 and TE-2 (30) were provided by Dr. Ari Ristimäki (Biomedicum, Helsinki, Finland). Cultures of foreskin-derived human fibroblasts, HFSF-132, were used from passages 7 to 15 as described elsewhere (31). All cells were cultured in RPMI 1640 (Life Technologies, Paisley, Scotland) supplemented with 10% fetal bovine serum (Life Technologies), 100 μg/mL streptomycin, and 100 units/mL penicillin.

Materials. The rabbit anti-c-Met antibodies and goat anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-phospho-Met (Y1234, Y1235) antibodies were from Upstate Biotechnology (Lake Placid, NY); and anti-human HGF/SF neutralizing antibody (AF-294-NA) were from R&D Systems (Minneapolis, MN). The ND_{50} for this lot of the antibody was in the range 0.2 to 0.6 μg/mL in the presence of 100 ng/mL of recombinant human HGF/SF. Acetylsalicylic acid, ibuprofen, indomethacin, diclofenac, and piroxicam were from Sigma (St. Louis, MO) and NS-398 was from Cayman Chemical (Ann Arbor, MI).

Preparation and collection of conditioned medium. The Bowes melanoma cell line was cultured for 3 days to reach its exponential growth phase. The culture medium was then changed and incubation prolonged for the next 48 hours. The conditioned medium was harvested and centrifuged (3,000 × *g*, 15 minutes) to sediment detached cells. Aliquots of the medium (500 μL) were applied to sparsely growing fibroblast cultures and incubation prolonged for 5 days.

Hepatocyte growth factor quantitation by ELISA. To determine HGF/SF concentration produced by cell cultures, the conditioned medium was harvested and the growth factor concentration estimated by the HGF/SF ELISA kit (R&D Systems) according to the manufacturer's protocol. Each sample was tested in triplicate by ELISA, and experiments were repeated thrice. The threshold sensitivity of the HGF/SF ELISA was 50 pg/mL.

Immunoblotting. Cell samples were lysed directly in SDS-PAGE sample loading buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 5% β-mercaptoethanol, and 0.005% bromophenol blue] supplemented with Complete Mini-protease inhibitor mixture tablets (Roche, Mannheim, Germany) and boiled for 5 minutes. Lysates were centrifuged at 14,000 rpm for 15 minutes to sediment particulate–insoluble material. The samples were separated in SDS-PAGE (gradient of polyacrylamide 5-15%, 3.5% stacking gel). The proteins were transferred electrophoretically from the gel to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), with transfer efficiency verified by Ponceau-S staining. After blocking of the membrane with 2.5% low-fat dry milk in TBS [20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween 20 (pH 7.5)], it was incubated with specific primary antibodies followed by an alkaline phosphatase–conjugated secondary antibody (Promega, Madison, WI). Protein bands were visualized according to manufacturer's recommendations. Band densities were quantified by densitometry.

Initiation and growth of spheroids. Multicellular spheroids were initiated and cultivated as described (25). U-bottomed 96-well plates (Costar, Cambridge, MA) were treated with 0.8% low-electroendosmotic agarose (Bio Whittaker, Rockland, ME) prepared in sterile water to form a thin film of nonadhesive surface. Fibroblasts were detached from culture dishes by trypsin/EDTA, and a single-cell suspension (4×10^4 cells/mL) was prepared in complete culture medium. To initiate spheroid formation, 250-μL aliquots were seeded into individual wells and the dishes incubated at 37°C in 5% CO_2 atmosphere.

Tumor spheroids were formed in a similar way except that 10^3 cells suspended in 250 μL of culture medium were seeded into agarose-treated 96-well plates. Suspended cells formed a single spheroid within 2 days of incubation, depending on the cell line. These spheroids were cultured for 5 days before employment for the invasion assay.

Scattering assay. Tumor cells were plated at a density of 10,000 cells per well into six-well plates. After cultivation for 3 days, cells were stimulated by the culture medium conditioned by fibroblast spheroids or control fibroblasts cultivated as monolayers for 96 hours. Cell scattering was evaluated under a phase-contrast microscope at 24 hours after stimulation.

Collagen invasion assay. Fifty spheroids were collected in 0.2 mL of culture medium and transferred into a mixture consisting of 0.8-mL collagen gel solution containing 1.3 mg/mL type I collagen (BD Biosciences, Bedford, MA), 0.8-mL 2-fold concentrated RPMI, and 0.2-mL FCS. The suspensions were poured into 35-mm bacteriologic plastic Petri dishes (BD Biosciences) and evenly spread by circular movements. The lattices with embedded spheroids were allowed to polymerize for 30 minutes at 37°C. Then 500-μL aliquots of medium conditioned by fibroblast spheroids for 96 hours or corresponding fibroblasts grown as monolayers were loaded onto the lattices. The extent of tumor cell outgrowth was scored by measurement at indicated time points of the major and minor axes of the spheroids by a phase-contrast microscope equipped with an ocular grid. Spheroid area was calculated and expressed as percentage of the initial area.

Effect of neutralizing anti-hepatocyte growth factor/scatter factor antibody. The effect of fibroblast-produced HGF/SF was examined by a neutralization procedure using goat anti-human HGF/SF antibody (R&D Systems) able to neutralize biological activity of 10 ng/mL HGF/SF at a concentration of 10 μg/mL. Before addition to melanoma or epithelial cell line cultures, the fibroblast-conditioned medium was incubated with anti-HGF/SF antibody for 2 hours. Normal goat serum IgG fraction purified with protein A-agarose (10 μg/mL) served as control.

Results

Culture medium conditioned by tumor cells induced hyperproliferation and aggregation of stromal fibroblasts. Our earlier results showed that tumor cells produce soluble factors mitogenic for stromal fibroblasts. Exceptionally potent growth-promoting activity was present in the conditioned medium of Bowes melanoma cell line (4). We now treated sparsely seeded monolayer cultures of human dermal fibroblasts by culture medium harvested from exponentially growing Bowes cells. Figure 1 shows the fibroblast morphology and growth pattern in response to culture medium conditioned by the tumor cells for 48 hours. Occasional hyperproliferative foci of fibroblasts began to be evident after 36 to 48 hours. Prolonged cultivation led to formation of multicellular aggregates growing upwards on the culture dish. After 120 hours, these clusters were tight and morphologically appeared as typical spheroid-like structures. Later on, these clusters detached from the culture dish surface into the culture medium, began to decompose, and finally only remnants floated there.

Hepatocyte growth factor abundantly produced by multicellular fibroblast spheroid clusters. Similar to induction by stimulation in tumor cell–conditioned medium, fibroblast spheroids can be effectively initiated as described (25). Conditioned medium from cultures of human fibroblast spheroids and corresponding monolayers was collected and quantified for HGF/SF by ELISA. Production of HGF/SF by fibroblast spheroids increased 220-fold at 48 hours compared with that detected in corresponding monolayer cultures (Fig. 2*A*). As shown in Fig. 2*A*, clustered fibroblasts produced up to 45 ng HGF/SF per 10^6 cells at 96 hours. An aliquot of the conditioned medium was separated by PAGE, and after immunoblotting, probed by specific anti-HGF/SF antibodies. Three bands with apparent M_r 80, 56, and 34 kDa were detectable in the spheroid-conditioned medium (Fig. 2*B*). As estimated by densitometric scanning of the immunoblot, the 80-kDa band represented 49%, the 56-kDa band represented 26%,

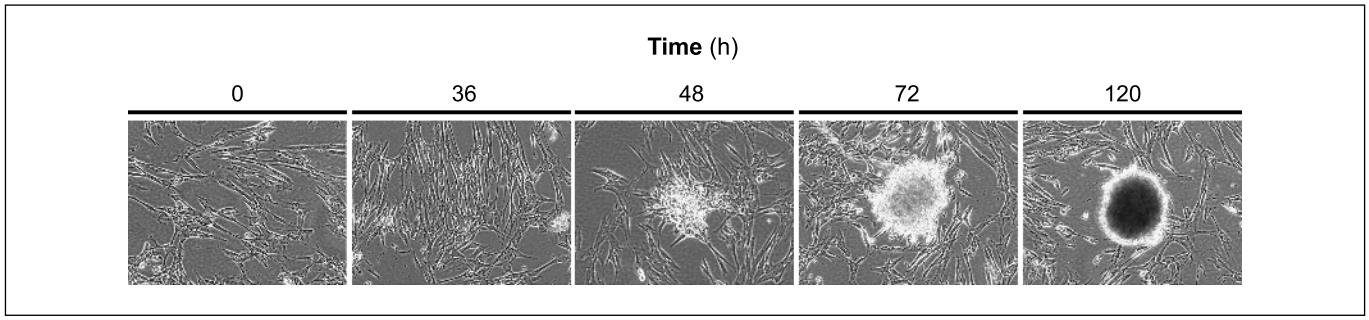

Figure 1. Formation of a multicellular fibroblast spheroid in response to tumor cell–conditioned medium. Addition of tumor cell–conditioned medium had a mitogenic effect on fibroblast monolayers. Briefly, at 0 hour, the culture medium of fibroblasts was replaced by a 48-hour conditioned medium from Bowes cell monolayers in their exponential growth phase. After 36 to 48 hours, occasional clustering foci of fibroblasts were evident. These multicellular clusters grew in size, with outgrowth of a multicellular cluster from the monolayer clearly apparent at 120 hours. All cells and cell lines were cultured in the same type of medium as described in Materials and Methods.

and the 34-kDa band represented 25%. This pattern indicates that at least half the HGF/SF produced was in its active processed form. None of the tumor cell lines selected for the study produced HGF/SF, and values measured in their conditioned medium were at the background level of ELISA (data not shown).

Expression of c-Met declined in fibroblasts spheroids. As for the expression kinetics of c-Met, the high-affinity receptor for HGF/SF, in fibroblast spheroids, three bands at 145, 170, and 190 kDa were evident in extracts of the stromal cells. The 145-kDa band corresponding to the mature β-chain of the receptor represented a major band, and the other two bands corresponded to the 190-kDa and 170-kDa precursor forms (Fig. 2*C*). As cell death and decomposition of the spheroids proceeded, evidenced by the time-dependent degradation of actin content evident in Fig. 2*C*, c-Met expression declined. In contrast, expression levels of c-Met remained unaltered for the 24 to 48 hours in control fibroblasts grown as monolayer cultures.

Expression of c-Met in selected tumor cell lines. Three melanoma cell lines, Bowes, WM-73, and WM-373, and two carcinoma cell lines, TE-1 and TE-2, were employed, because they expressed c-Met differently (Fig. 3*A*). In the set of melanoma cell lines, the fully processed 145-kDa c-Met form was detected in Bowes cells, whereas WM-373 expressed the 190-kDa unprocessed form, and because a barely detectable signal for c-Met appeared there, the WM-75 represented a negative control. Both epithelial cancer cell lines TE-1 and TE-2 were positive for c-Met expression, because the

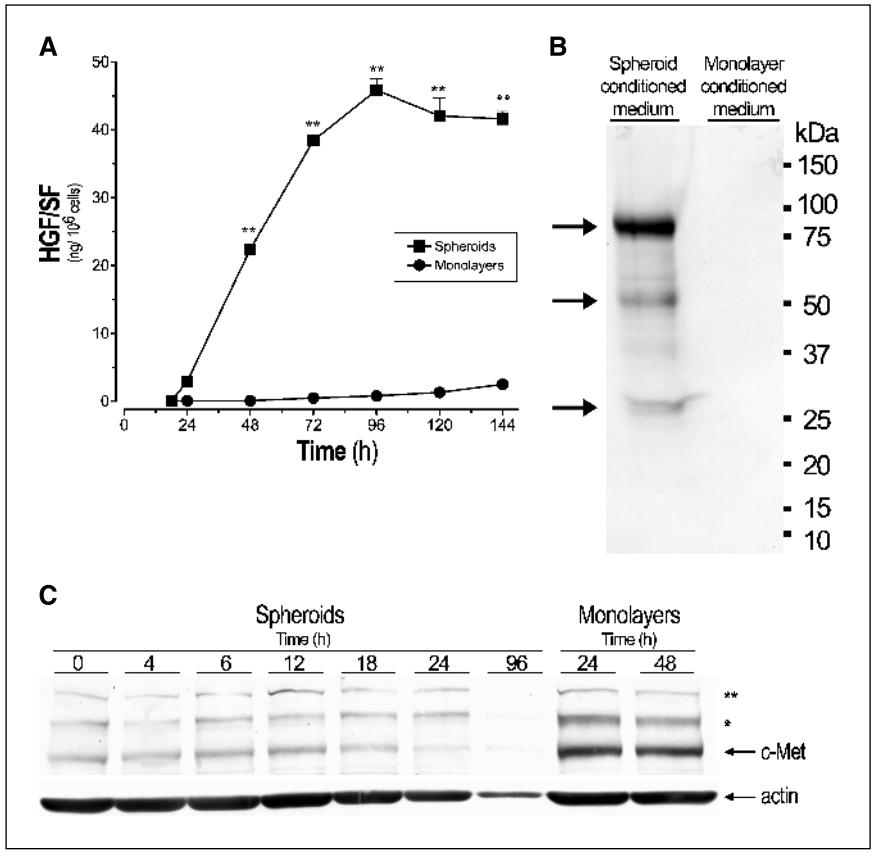

Figure 2. *A,* production of HGF/SF by spheroids and corresponding monolayer cultures. Concentrations of HGF/SF were measured from conditioned medium of cells cultured concurrently as three-dimensional spheroids or monolayers. **, $P < 0.001$, compared with corresponding monolayers. *Points,* means from three independent experiments; *bars,* ±SE. *B,* analysis of HGF/SF molecular weight species as produced by spheroids and monolayers of fibroblasts. Three different molecular weight species of HGF/SF were detectable in immunoblot analysis of conditioned medium from spheroid cultures. *Arrows,* ~80-kDa mature HGF/SF, and the HGF/SF heavy and light chains at 60 and 30 kDa, respectively. The corresponding conditioned medium from monolayer cultures was negative for HGF/SF by immunoblotting. Conditioned medium from both spheroid and monolayer cultures of fibroblasts was collected after 96 hours of culture. *C,* down-regulation of the HGF/SF receptor, c-Met, in multicellular fibroblast spheroids. Expression of c-Met in multicellular spheroids diminished during progression of decomposition and cell death. No change occurred in c-Met expression in corresponding monolayer cultures. *Asterisks,* partly processed precursor forms of c-Met. Cells were cultured as spheroids and corresponding monolayers, collected at indicated time points, and processed for immunoblot analysis. Actin content served as a representative intracellular control component of protein loading. Experiments were done in triplicate, and the figure shows data from one representative experiment.

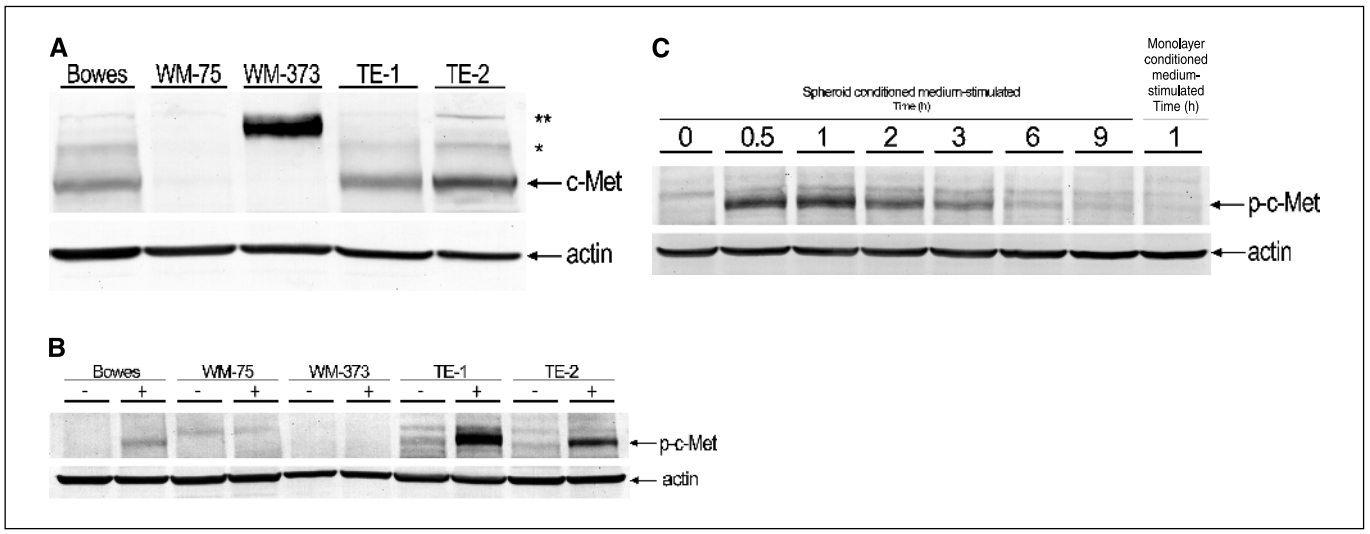

Figure 3. *A,* expression of c-Met and its precursors in melanoma and epithelial cancer cell lines. The melanoma cell lines Bowes, WM-75, and WM-373 differ in their expression pattern of mature and precursor forms of c-Met. No expression of mature and fully processed c-Met occurred in WM-75 and WM-373, whereas Bowes cells predominantly expressed this form. A dominant band (*asterisks*) corresponding to the unprocessed c-Met precursor protein is apparent in WM-373 cell lysates. Both the epithelial cancer cell lines TE-1 and TE-2 expressed fully processed c-Met. In both, precursor forms (*asterisks*) of c-Met were also detectable, predominantly in TE-2. *B,* phosphorylation of c-Met in melanoma and epithelial cell lines in response to conditioned medium from spheroids and corresponding monolayers. Monolayers of melanoma and epithelial cancer cell lines were treated for 1 hour with the 96-hour conditioned medium from multicellular fibroblast spheroids (+) or corresponding monolayer cultures (−). The phosphorylated c-Met (p-c-Met) detected corresponded to the c-Met expression pattern as shown in (*A*). In response to spheroid-conditioned medium, increased phosphorylation of c-Met was detectable in Bowes, TE-1, and TE-2 cell lines. The c-Met-negative cell lines WM-75 and WM-373 showed no increase in c-Met phosphorylation when treated with spheroid-conditioned medium compared with that of corresponding monolayer control. Actin content is presented as confirmation of equal protein loading of lanes. The most distinct phosphorylation of c-Met appeared in the epithelial cell lines TE-1 and TE-2 treated with spheroid-conditioned medium. *C,* time course of c-Met phosphorylation in response to spheroid-conditioned medium in TE-1 cells. Ninety-six-hour conditioned medium was collected from both spheroid and monolayer cultures of human dermal fibroblasts. TE-1 cell monolayers were stimulated with the conditioned medium for indicated time points, with samples collected for immunoblot analysis. Phosphorylation of c-Met was evident already at 0.5 to 1 hour of stimulation with spheroid-conditioned medium. This response was rapid and transient and was not evident in corresponding monolayer controls.

processed 145-kDa receptor protein (Fig. 3*A*) was clearly detectable. Figure 3*A* represents an immunoblot of tumor cells grown as multicell spheroids. An identical pattern was detectable upon probing of cell lysates from monolayers (data not shown).

Conditioned medium from fibroblast spheroids induced phosphorylation of c-Met in tumor cells. To analyze the action of HGF/SF produced by the fibroblast spheroids, the tumor cell lines, grown as monolayers, were treated simultaneously for 1 hour with medium conditioned for 96 hours by spheroid cultures and corresponding standard cultures. Figure 3*B* shows the phosphorylation pattern of c-Met in the tumor cells. The cells expressing the processed 145-kDa c-Met form (Bowes, TE-1, and TE-2), after stimulation by the conditioned medium harvested from fibroblast spheroids, contained phosphorylated c-Met. No such signal was detectable in the c-Met-negative WM-75 cell line or in WM-373 cells with unprocessed c-Met. In fact, a slight decline in phosphorylation of the unprocessed c-Met in WM-75 cells was evident. Moreover, the unprocessed c-Met intensively expressed in WM-373 cells (as shown in Fig. 3*A*) showed no phosphorylation response to conditioned medium from either fibroblast monolayer or spheroid cultures.

To investigate further the phosphorylation kinetics of c-Met in tumor cells, we selected the TE-2 cell line showing a clearly increased phosphorylation at 1 hour (Fig. 3*B*). TE-2 cells were treated with the fibroblast spheroid–conditioned medium, with samples collected at the time points indicated in Fig. 3*C*. By 0.5 hour, a rapid increase in c-Met phosphorylation was already evident in these cells. However, this increase proved to be transient, because after 2 hours, the degree of phosphorylation began to decline, attaining at 9 hours levels similar to those of the fibroblast monolayer–conditioned medium–treated cells.

Conditioned medium from fibroblast spheroids promoted tumor cell scattering. To show a biological response to the HGF/SF produced by the multicellular fibroblast aggregates, we tested the scattering response of the melanoma and carcinoma cell lines. Figure 4 shows cell morphology at 24 hours after treatment with either fibroblast monolayer or spheroid culture 96-hour conditioned medium. The cell lines (Bowes, TE-1, and TE-2) positive for processed c-Met expression and c-Met phosphorylation responded to spheroid-conditioned medium by a flattening and scattering of cells. Distinct stellar morphology and formation of long, prominent cellular processes visible in Bowes and TE-1 cell lines suggests increased cell motility in response to spheroid-conditioned medium.

Hepatocyte growth factor/scatter factor produced by fibroblast spheroids for enhancement of tumor cell motility and invasiveness. Tumor cells are viable and are able to maintain a proliferative state as multicellular spheroids. Tumor spheroids frequently serve as an *in vitro* model system to mimic nodular tumor growth (22). We preformed spheroids from the tumor cell lines and thereafter embedded them in a collagen lattice. Once the collagen was polymerized (after 30 minutes), conditioned medium from either fibroblast monolayer or spheroid cultures was poured into cultures and the incubation prolonged for 3 days. Cellular outgrowth from the tumor spheroids was assessed with an ocular millimeter grid, with measurement of the area of the cultures corresponding to total outgrowth of the tumor spheroids. Figures 5 and 6 show the outgrowth kinetics of the melanoma and epithelial tumor cell lines.

The Bowes cell spheroids expressing the fully processed and functional c-Met attached rapidly to the lattice, and even the control spheroids flattened and began to spread. In the fibroblast spheroid–conditioned medium–treated Bowes spheroids, however, a clear 3.7-fold increase in cell outgrowth (measured as total area of

the culture) from the spheroid had already occurred at 18 hours (Fig. 5*A*). In these cultures, the growth-stimulatory effect of fibroblast spheroid–conditioned medium remained until the end of the experiment (after 72 hours). The WM-75 melanoma cell spheroids with low levels of c-Met, showing no phosphorylation in response to fibroblast spheroid–conditioned medium, spread only minimally on the surface in response to either treatment. A similar response occurred in the WM-373 cell spheroids, which expressed the unprocessed c-Met. Interestingly, both these melanoma cell spheroids with low or defective c-Met signaling responded with less outgrowth when stimulated with fibroblast spheroid–conditioned medium than did the monolayer control at 30 hours for WM-75 and after 48 hours for WM-373 (Fig. 5*B* and *C*).

The epithelial cancer cell lines TE-1 and TE-2, shown to express c-Met with functional phosphorylation, in terms of cell scattering, to fibroblast spheroid–conditioned medium, both also responded with increased cell outgrowth from the spheroids (Fig. 6). Although a more rapid response occurred in TE-1 spheroids (Fig. 6*A*), the slower-responding TE-2 cells showed a 10-fold increase in outgrowth area at 72 hours in response to fibroblast spheroid–conditioned medium compared with control (Fig. 6*B*). We used this clear response of the TE-2 spheroids to ascertain that the tumor progression–promoting activity related to HGF/SF in the fibroblast spheroid–conditioned medium. Conditioned media were pretreated with an anti-HGF/SF–neutralizing antibody before assay of their activity on tumor cells. We observed that the antibody effectively blocked the motile response of

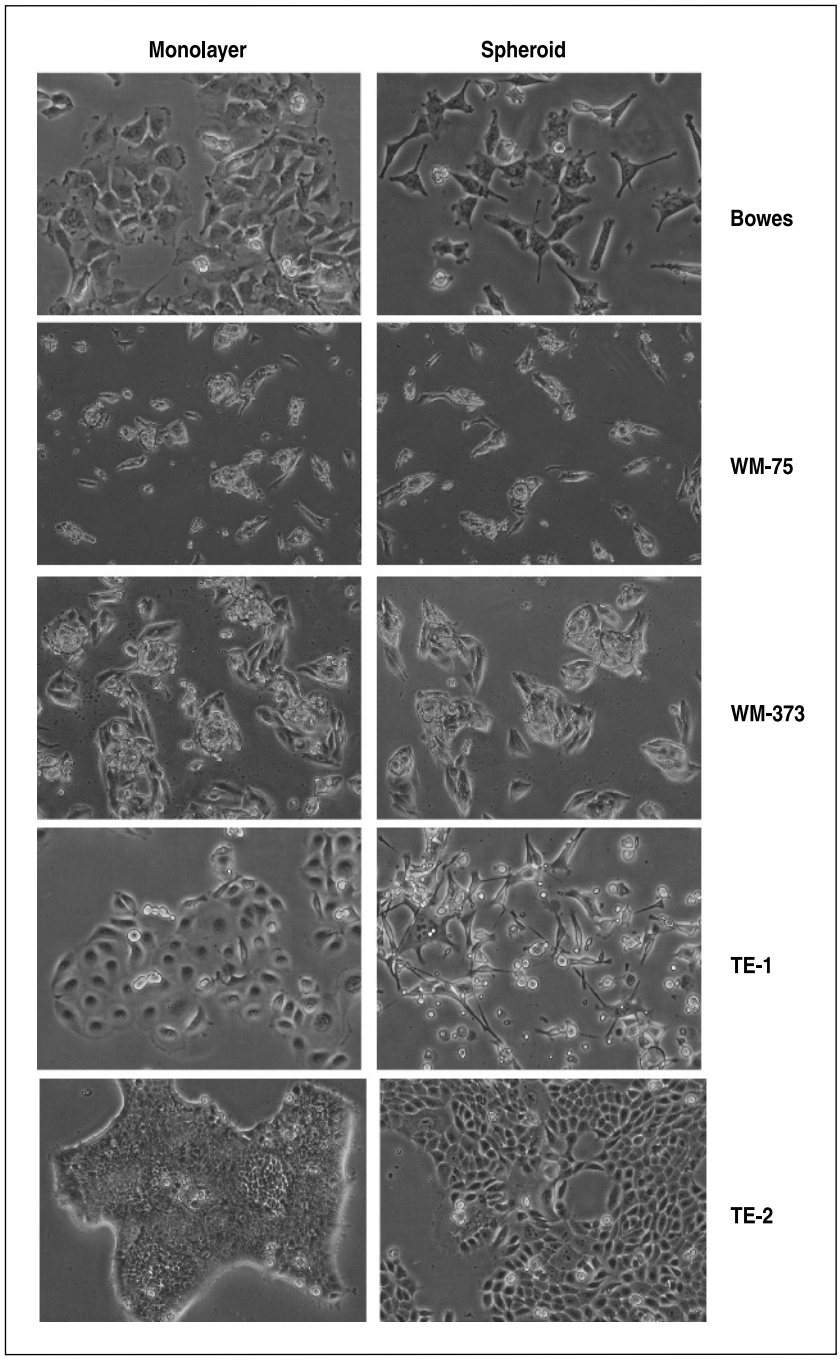

Figure 4. Scattering of tumor cells influenced by conditioned medium from fibroblast spheroids or corresponding monolayer cultures. Culture medium of exponentially growing monolayer cultures of indicated cell lines was replaced by the 96-hour conditioned medium from control monolayer or multicellular spheroid cultures of human dermal fibroblasts. Cell morphology for scattering was estimated after 24 hours. In response to conditioned medium from spheroid cultures of fibroblasts, Bowes, TE-1, and TE-2 cells showed a clear response in terms of cell morphology change, whereas the morphology of WM-75 and WM-373 cells remained unchanged. Spheroid-conditioned medium–affected Bowes and TE-1 cells became stellar shaped and developed protrusions. The spheroid-conditioned medium–affected TE-2 cells became larger in size with more distinct cell-cell borders.

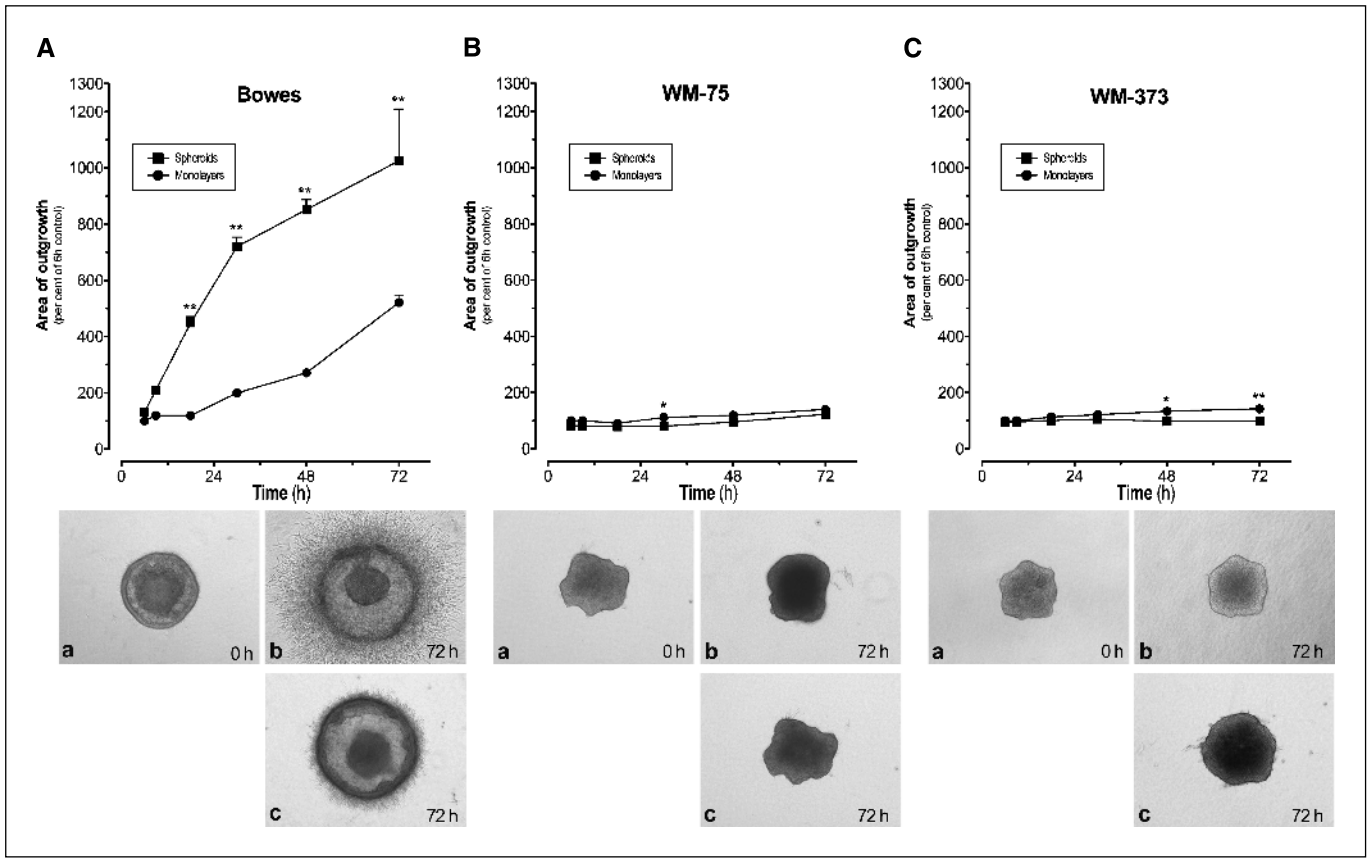

Figure 5. Outgrowth of melanoma cells from spheroid cultures treated with conditioned medium from fibroblast spheroids and corresponding monolayer cultures. Spheroid cultures of melanoma cells were preformed for 48 hours. Their culture medium was replaced at 0 hour with the 96-hour conditioned medium from fibroblast spheroids or corresponding monolayers. The area of outgrowth of cells from the tumor spheroids was measured by microscopy with a millimeter grid and was followed for the indicated times. *A,* Bowes cells, showing increased phosphorylation of c-Met when treated with spheroid-conditioned medium, responded to fibroblast spheroid–conditioned medium with aggravated outgrowth evident from 18 hours. A 97% increase in outgrowth area occurred in fibroblast spheroid–conditioned medium–treated cultures at 72 hours compared with the corresponding monolayer-conditioned medium–treated controls. *B,* outgrowth of WM-75 cells from spheroid cultures was not altered between spheroid or control monolayer-conditioned medium–treated cells. A significant but transient 39% increase in outgrowth occurred at 30 hours in the monolayer control-treated cells compared with the fibroblast spheroid–treated cells. *C,* outgrowth of WM-373 cells treated with control monolayer-conditioned medium was significantly greater at 48 to 72 hours than in fibroblast spheroid–treated cultures. Outgrowth from monolayer-conditioned medium–treated tumor spheroids attained an increase of 44% at 72 hours. *Lower panels: a,* control tumor spheroid at 0 hour; *b*, fibroblast spheroid–conditioned medium–treated tumor spheroid at 72 hours; *c,* control monolayer-conditioned medium–treated tumor spheroid at 72 hours. *Points,* means of percentage of 6-hour control values collected from four to six independent experiments; *bars,* ±SE. *, $P < 0.01$; **, $P < 0.001$, compared with the corresponding control.

tumor cells incubated with spheroid-conditioned medium (Fig. 6*B*). A 70% decrease in the invasive capacity of the cells occurred in the presence of spheroid-conditioned medium pretreated with the neutralizing anti-HGF/SF antibody. The values with the antibody were similar to those with control fibroblast monolayer–conditioned medium (data not shown).

Nonsteroidal anti-inflammatory drugs attenuated hepatocyte growth factor/scatter factor production by fibroblast spheroids. We previously found (25) that cyclooxygenase-2 (COX-2)–inhibiting NSAIDs, such as NS-398 and indomethacin, are able to inhibit the programmed cell death pathway and release of lactate dehydrogenase from cells in the dermal fibroblast spheroids. By this rationale, we tested their effect on production of HGF/SF. Six NSAIDs (acetylsalicylic acid, diclophenac, ibuprofen, indomethacin, NS-398, or piroxicam) were added at the time of cell seeding to the fibroblast spheroid cultures, and concentrations of HGF/SF were measured at 96 hours and compared with the vehicle-treated controls. All the NSAIDs concentration dependently inhibited production of HGF/SF in the fibroblast spheroids in the following IC_{50} order: NS-398 (119 μmol/L) < diclophenac (144 μmol/L) < indomethacin (219 μmol/L) < piroxicam (465 μmol/L) < ibuprofen (729 μmol/L) < acetylsalicylic acid (3720 μmol/L). To investigate how much this inhibitory effect depended on the extent of spheroid formation, we added IC_{50} concentrations of NS-398 or indomethacin to the cultures at different time points after seeding of the spheroids, and HGF/SF production was measured at 96 hours as previously. When they were added at 4 hours or before, the inhibitory effects of both NS-398 and indomethacin remained at the control level (Fig. 7). Thereafter, these drugs' efficacy in inhibiting HGF/SF production declined, and with addition at 24 hours (formation of spheroids completed) or thereafter, their inhibitory effects were abolished suggesting that only administration at early stages of multicellular aggregate formation can prevent HGF/SF production.

Discussion

Our data suggest that proliferative signals from cancer cells induce the fibroblast hyperproliferation and aggregation associated with commitment to a previously identified nonapoptotic cell death

pathway (25). We now show that one of the crucial elements in tumor progression (i.e., signaling by the receptor tyrosine kinase c-Met) is activated by HGF/SF derived from the fibroblast aggregates. Paracrine signaling can thus stimulate the formation of a reciprocal activating loop causing alterations in gene expression, cell morphology, and behavior, leading to a more malignant phenotype of the transformed cancer cells (32). The key players involved in this type of signaling have largely been identified (20, 33). Although some degree of modulation over HGF/SF production can be achieved by cytokines such as interleukin-1 or tumor necrosis factor-α (34), a cascade to induce production in high concentrations within the tumor microenvironment has remained unshown.

Mutations rendering the receptor tyrosine kinase overtly active or defective processing of c-Met in epithelial tumor cells (18), harboring the predominant expression of c-Met compared with stromal cells (35), can explain, at least in part, the differences in kinetics of cancer cell growth (19). Because most interest has focused on c-Met on tumor cells, little is known about the production and expression of HGF/SF in tumor-associated stromal cells. Here, we considered solid tumors as representing cellular systems maintained by dynamic interplay between neoplastic cells and stromal fibroblasts. This concept is further supported by fibroblast-driven acceleration of tumor growth *in vivo* (36). Supporting our previous results on tumor cell–derived factors stimulating fibroblast growth (4), prolonged stimulation by tumor cell conditioned medium led to local hyperproliferation of fibroblasts and to occasional formation of very dense multicellular clusters detaching from the culture dish. No such process was evident in control fibroblasts grown as monolayers with contact inhibition operating. In a three-dimensional culture of fibroblast multicellular aggregates, we recently observed that the cells were committed to a novel cell death pathway (25). As similar clusters were formed by stimulation with tumor cell–derived factors, we here adopted the spheroid model to further investigate paracrine signaling initiated by cell-cell contacts in stromal fibroblasts. Certain types of tumor cells (e.g., melanoma) have a tendency to grow spontaneously as multicell clusters in standard monolayer culture (see Fig. 4 for growth pattern of WM-75 and WM-373 cells). In contrast to fibroblasts, the clustered tumor cells exhibited progressive growth, suggesting that different control mechanisms operate in three-dimensional arrangements of normal or tumor cells.

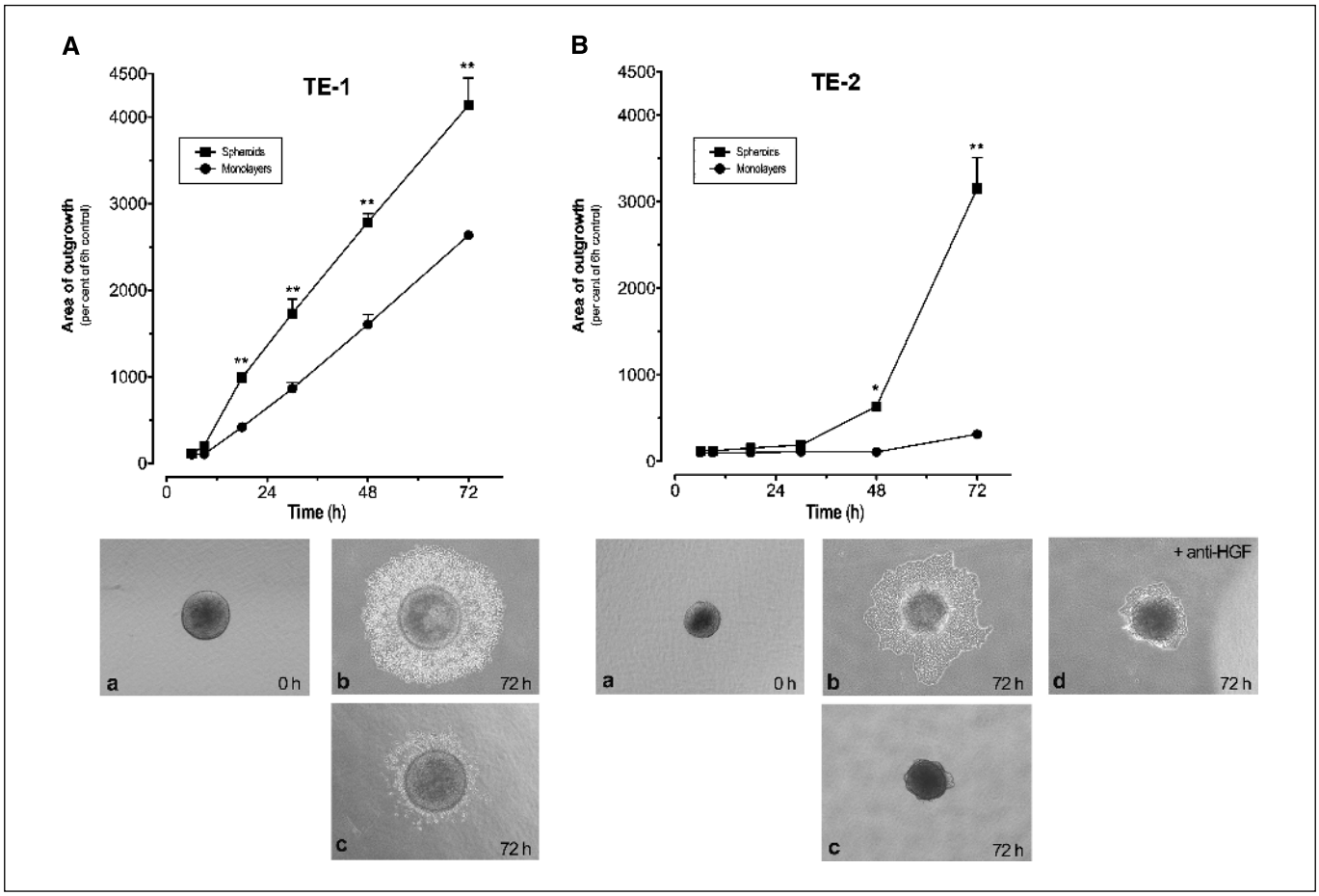

Figure 6. Outgrowth of epithelial cells from spheroid cultures treated with conditioned medium from fibroblast spheroids and corresponding monolayer cultures. Spheroids of the epithelial cell lines TE-1 and TE-2 were preformed for 48 hours. Their culture medium was replaced at 0 hour with the 96-hour conditioned medium from fibroblast spheroids or corresponding monolayers. The area of outgrowth of cells from the tumor spheroids was calculated by microscopy with a millimeter grid and was followed for the times indicated. *A,* TE-1 cells responded to fibroblast spheroid–conditioned medium with aggravated outgrowth starting from 18 hours. At 72 hours, a 57% increase in the area of outgrowth was seen. *B,* outgrowth of TE-2 cells from spheroid cultures was slow, beginning at 48 hours, and reaching a 900% increase at 72 hours in the fibroblast spheroid–conditioned medium–treated cells. *Lower panels: a,* control tumor spheroid at 0 hour; *b,* fibroblast spheroid–conditioned medium–treated tumor spheroid at 72 hours; *c,* control monolayer-conditioned medium–treated tumor spheroid at 72 hours. *B,* a 72-hour control experiment (*d*) showed the effect of a function-blocking anti-HGF/SF antibody on fibroblast spheroid–conditioned medium–treated TE-2 tumor cell outgrowth. *Points,* means of percentage of 6-hour control values collected from four to six independent experiments; *bars,* ±SE. *, $P < 0.01$; **, $P < 0.001$, compared with corresponding control.

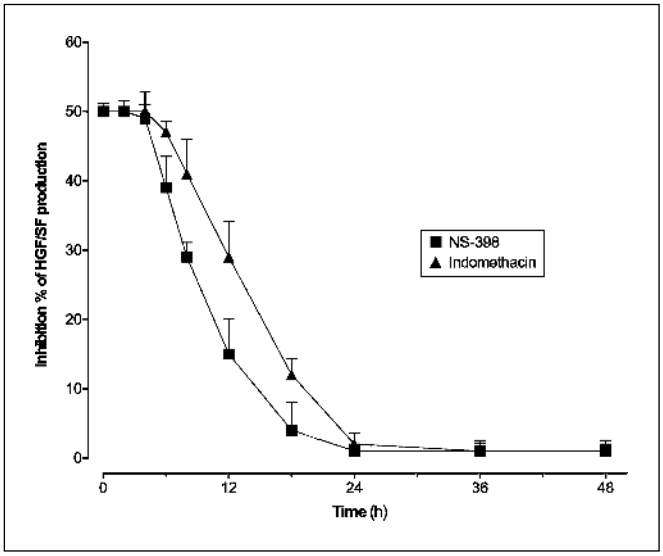

Figure 7. Time dependence and efficacy of inhibition of HGF/SF production by NS-398 and indomethacin added at the indicated time points after formation of fibroblast spheroids. IC_{50} concentrations of NS-398 (100 μmol/L) or indomethacin (300 μmol/L) were added to the forming fibroblast spheroid cultures at the indicated time points, with production of HGF/SF evaluated at 96 hours and compared with their respective vehicle-treated controls. The inhibitory efficacy of both drugs on HGF/SF production clearly began to decline when the drugs were added after 6 hours of spheroid formation. Addition of the drugs at 24 hours or thereafter produced no inhibition of HGF/SF production by the fibroblast spheroid cultures. *Points,* means from three independent experiments; *bars,* ±SE.

We show here that the production of HGF/SF was superinducible by homotypic cell-cell contacts and was associated with a novel type of nonapoptotic fibroblast cell death and necrosis-like morphology. The estimated concentration of HGF/SF in conditioned medium represented up to 45 ng per 10^6 cells at 96 hours. Such abundant production of the growth factor seems never to have been reported to be induced in human fibroblasts by any stimuli. Importantly, no HGF/SF was detected in our tumor cell lines cultured either as monolayers or spheroids. HGF/SF is ubiquitously present in plasma and in the extracellular matrix of tissues, where it accumulates, due to its affinity for proteoglycans (37), as an inactive precursor (38). We found that almost half the HGF/SF produced by the cell-cell contact–activated fibroblasts is in its bioactive form. As cell death and consequent decomposition of fibroblasts within clusters were accompanied by enhanced proteolytic activation of plasminogen (25), it seems possible that the urokinase-mediated proteolytic system may serve to activate the HGF/SF precursor form (39, 40).

The biological actions of the active form of HGF/SF are mediated by a single membrane-bound high-affinity receptor, c-Met. The Bowes melanoma cell line, as well as the two carcinoma cell lines TE-1 and TE-2, expressing processed c-Met, responded to conditioned medium from fibroblast spheroids by greater scattering and invasiveness than to the corresponding monolayer-conditioned medium. These responses paralleled increases in c-Met phosphorylation. By use of the TE-2 cell line, we showed this phosphorylation response to be rapid and transient, whereas its downstream effects in responsive cells lasted over 3 days. In contrast, those melanoma cell lines WM-75 or WM-373, expressing undetectable or unprocessed c-Met, respectively, remained unresponsive to HGF/SF in the culture medium of mesenchymal spheroids, showing the requirement of a functional c-Met for proper HGF/SF responsiveness and providing further evidence that the factor released from the spheroid cultures is indeed HGF/SF.

The presence of necrotic foci and inflammation within a tumor mass is commonly associated with poor prognosis and with the aggravated nature of brain tumors and breast and colorectal cancers (27, 33). Taken together with our earlier findings of commitment to programmed cell death with necrotic morphology and induction of COX-2 (25), superinduced production of HGF/SF provides a molecular and morphologic rationale for enhanced tumor progression by tumor-stroma interaction in the presence of inflammation and necrosis.

As the COX-2-inhibiting NSAIDs exert cancer-preventive effects (29, 41), we tested their efficacy in inhibiting HGF/SF production induced in fibroblast spheroids. These NSAIDs concentration dependently inhibited HGF/SF release from the spheroid aggregates. Interestingly, the NSAIDs' inhibitory effect occurred only upon administration before formation of cell-cell contacts and full commitment to the cell-death pathway. When drug treatments were delayed for >24 hours, full-scale induction of HGF/SF occurred comparable with that of untreated control cultures. In short, this suggests a very early contribution of prostaglandin production within fibroblast spheroids to initiation of the cascade leading to HGF/SF production. We can speculate that this effect shows that only prolonged NSAID administration will show any tumor-preventive effect and thus will produce timely inhibition of cell-cell contact–induced fibroblast activation, because no specific marker for this phenomenon yet exists.

Superinduction of HGF/SF by cell-cell contacts of fibroblasts, together with our previously reported induction of COX-2 and plasminogen activation, suggests that this type of nonapoptotic cell death with necrotic morphology in fibroblast commitment and activation is a novel type of preprogrammed cascade. We have described this phenomenon as programmed necrosis (25), but as it deserves a more unique identification among a growing array of types of cell death, we propose the description "nemosis," based on the Greek goddess of retribution and inevitable consequence, Nemesis. Her maintaining equilibrium by punishing immoderate actions is like that of fibroblast spheroids: any greatly enhanced production of mediators linked to organogenesis, cancer, or inflammation unavoidably results in cell death.

As in nemosis, interrelations between HGF/SF, c-Met, and COX-2 and the plasminogen activation cascade are further strengthened by recent observations of such interactions in the tumor-associated thrombohemorrhagic phenotype (42). Moreover, all these mediators may be involved in tumor "nesting" or in formation of fibrin matrix as a prerequisite for tumor growth and neoangiogenesis (43).

Here, we show apparently for the first time, that fibroblasts, when cultured as clusters, express a distinct pattern of cellular decomposition accompanied by superinduced production of HGF/SF. This suggests that within a tumor the programmed necrotic/nemotic death of mesenchymal cells such as stromal fibroblasts constitutes a principal source of the active HGF/SF required for efficient tumor cell migration and metastasis. Moreover, because HGF/SF production was attenuated only by early treatment of the clustering cells with NSAIDs, an alternative mechanism by which long-term NSAIDs may assist in modulation of tumorigenesis and tumor progression may be via inhibition of the HGF/SF produced by nemotic foci of dying fibroblasts.

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References

1. Elenbaas B, Weinberg RA. Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. Exp Cell Res 2001;264:169–84.

2. Ruiter D, Bogenrieder T, Elder D, Herlyn M. Melanoma-stroma interactions: structural and functional aspects. Lancet Oncol 2002;3:35–43.

3. Houghton AN. LIGHTing the way for tumor immunity. Nat Immunol 2004;5:123–4.

4. Lizonova A, Bizik J, Grofova M, Vaheri A. Coexpression of tumor-associated α2-macroglobulin and growth factors in human melanoma cell lines. J Cell Biochem 1990;43:315–25.

5. Cornil I, Theodorescu D, Man S, Herlyn M, Jambrosic J, Kerbel RS. Fibroblast cell interactions with human melanoma cells affect tumor cell growth as a function of tumor progression. Proc Natl Acad Sci U S A 1991; 88:6028–32.

6. Sappino AP, Skalli O, Jackson B, Schurch W, Gabbiani G. Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues. Int J Cancer 1988;41:707–12.

7. Ronnov-Jessen L, Petersen OW, Bissell MJ. Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol Rev 1996;76:69–125.

8. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature 2004;432:332–7.

9. Labrousse AL, Ntayi C, Hornebeck W, Bernard P. Stromal reaction in cutaneous melanoma. Crit Rev Oncol Hematol 2004;49:269–75.

10. Stoker M, Gherardi E, Perryman M, Gray J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature 1987;327:239–42.

11. Montesano R, Matsumoto K, Nakamura T, Orci L. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell 1991;67: 901–8.

12. Nakamura T, Matsumoto K, Kiritoshi A, Tano Y, Nakamura T. Induction of hepatocyte growth factor in fibroblasts by tumor-derived factors affects invasive growth of tumor cells: *in vitro* analysis of tumor-stromal interactions. Cancer Res 1997;57:3305–13.

13. Bertotti A, Comoglio PM. Tyrosine kinase signal specificity: lessons from the HGF receptor. Trends Biochem Sci 2003;28:527–33.

14. Zhang YW, Vande Woude GF. HGF/SF-Met signaling in the control of branching morphogenesis and invasion. J Cell Biochem 2003;88:408–17.

15. Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Vande Woude G. Sequence of MET protooncogene cDNA has features characteristics of the tyrosine family of proto-factor receptors. Proc Natl Acad Sci U S A 1987;84:6379–83.

16. Jin L, Fuchs A, Schnitt SJ, et al. Expression of scatter factor and c-Met receptor in benign and malignant breast tissue. Cancer 1997;79:749–60.

17. Jiang WG, Hiscox S, Matsumoto K, Nakamura T. Hepatocyte growth factor/scatter factor, its molecular, cellular and clinical implications in cancer. Crit Rev Oncol Hematol 1999;29:209–48.

18. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. Nat Rev Mol Cell Biol 2003;4:915–25.

19. Ma PC, Maulik G, Christensen J, Salgia R. c-Met: structure, functions and potential for therapeutic inhibition. Cancer Metastasis Rev 2003;22:309–25.

20. Tamagnone L, Comoglio PM. Control of invasive growth by hepatocyte growth factor (HGF) and related scatter factors. Cytokine Growth Factor Rev 1997;8: 129–42.

21. Sutherland RM, McCredie JA, Inch WR. Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. J Natl Cancer Inst 1971;46: 113–20.

22. Sutherland RM. Cell and environment interactions in tumor microregions: the multicell spheroid model. Science 1988;240:177–84.

23. Kandel J, Bossy-Wetzel E, Radvanyi F, Klagsbrun M, Folkman J, Hanahan D. Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell 1991;66:1095–104.

24. Frisch SM, Screaton RA. Anoikis mechanisms. Curr Opin Cell Biol 2001;13:555–62.

25. Bizik J, Kankuri E, Ristimäki A, et al. Cell-cell contacts trigger programmed necrosis and induce cyclooxygenase-2 expression. Cell Death Differ 2004;11: 183–95.

26. Gilchrist KW, Gray R, Fowble B, Tormey DC, Taylor SG 4th. Tumor necrosis is a prognostic predictor for early recurrence and death in lymph node-positive breast cancer: a 10-year follow-up study of 728 Eastern Cooperative Oncology Group patients. J Clin Oncol 1993;11:1929–35.

27. Vakkila J, Lotze MT. Inflammation and necrosis promote tumor growth. Nat Rev Immunol 2004;4:641–8.

28. Thun MJ, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. J Natl Cancer Inst 2002;94:252–66.

29. Turini ME, DuBois RN. Cyclooxygenase-2: a therapeutic target. Annu Rev Med 2002;53:35–57.

30. Fujiwara Y, Sugita Y, Nakamori S, et al. Assessment of stanniocalcin-1 mRNA as a molecular marker for micrometastasis of various human cancers. Int J Oncol 2000;16:799–804.

31. Bizik J, Bessou S, Felnerova D, Vaheri A, Taieb A. The proteolytic potential of normal human melanocytes: comparison with other skin cells and melanoma cell lines. Pigment Cell Res 1996;9:255–64.

32. Kinzler KW, Vogelstein B. Landscaping the cancer terrain. Science 1998;280:1036–7.

33. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420:860–7.

34. Tamura M, Arakaki N, Tsoubouchi H, Takada H, Dalkuhara Y. Enhancement of human hepatocyte growth factor production by interleukin-1 α and -1 β and tumor necrosis factor-α by fibroblasts in culture. J Biol Chem 1993;268:8140–5.

35. Gonzatti-Haces M, Seth A, Park M, Copeland T, Oroszlan S, Vande Woude GF. Characterization of the TPR-MET oncogene p65 and the MET protooncogene p140 protein tyrosine kinases. Proc Natl Acad Sci U S A 1988;85:21–5.

36. Camps JL, Chang S, Hsu TC, et al. Fibroblast-mediated acceleration of human epithelial tumor growth *in vivo*. Proc Natl Acad Sci U S A 1990;87:75–9.

37. Lyon M, Deakin JA, Mizuno K, Nakamura T, Gallagher JT. Interaction of hepatocytes growth factor with heparan-sulfate. Elucidation of the major heparan sulfate structural determinants. J Biol Chem 1994;269: 11216–23.

38. Miyazawa K, Shimomura T, Naka D, Kitamura N. Proteolytic activation of hepatocyte growth factor in response to tissue injury. J Biol Chem 1994;269:8966–70.

39. Naldini L, Vigna E, Bardelli A, Follenzi A, Galimi F, Comoglio PM. Biological activation of pro-HGF (hepatocyte growth factor) by urokinase is controlled by a stoichiometric reaction. J Biol Chem 1995;270:603–11.

40. Myöhänen H, Vaheri A. Regulation and interactions in the activation of cell-associated plasminogen. Cell Mol Life Sci 2004;61:2840–58.

41. Warner TD, Mitchell JA. Cyclooxygenases: new forms, new inhibitors, and lessons from the clinic. FASEB J 2004;18:790–804.

42. Boccaccio C, Sabatino G, Medico E, et al. The MET oncogene drives a genetic programme linking cancer to haemostasis. Nature 2005;434:396–400.

43. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med 1986;315:1650–9.