

Role of the MET/HGF receptor in proliferation and invasive behavior of osteosarcoma

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ABSTRACT

Signal transduction downstream HGF receptor (MET) activation involves multiple pathways that account for mitogenesis, motility and morphogenesis in a cell type-dependent fashion. MET receptor is aberrantly expressed in almost 100% of human osteosarcomas. We analyzed the effect of the MET receptor activation in five human osteosarcoma cell lines evaluating the levels of HGF-dependent activation of MAPK and PKB/AKT as biochemical readouts of mitogenic and invasive responses, respectively. All the cell lines tested expressed high levels of the *MET* proto-oncogene. Four cell lines showed activation of the MAPK cascade upon HGF stimulation, suggesting that this growth factor serves a common proliferative function in osteosarcomas. Two lines showed activation of PKB/AKT, that is known to be involved in migration mediated by HGF receptor. Accordingly, cell lines where MAPK cascade was activated responded to HGF with increased proliferation, while induction and inhibition of PKB/AKT activity corresponded to acquisition or block of the invasive-motile response to HGF, respectively. Both the HGF dependent responses were reverted by the specific MET inhibitor K252a. These data show that HGF activates both the mitogen and motility machinery in osteosarcoma cells and suggest that HGF might promote their malignant behavior by concomitant activation of different pathways and biological functions.

Key words: signal transduction • MAPK • PKB/AKT • invasion • tumor growth

The hepatocyte growth factor (HGF/SF) receptor MET regulates mitogenesis, motility, and morphogenesis in a cell type-dependent fashion. These different behaviors are part of a single complex phenomenon, termed invasive growth, which is involved in several physiological processes. *In vivo*, either *MET* or *HGF* gene deletion results in impairment of myoblast migration and neuron outgrowth (for a review, see ref 1). During morphogenesis *in vitro*, MET activation results in the formation of tubular structures in specific cell types such as liver progenitor cells. HGF/SF is also a strong angiogenic factor both *in vivo* and *in vitro* (2, 3) and shows wound healing activity. Deregulated activation of HGF-stimulated pathways

contributes to cancer onset and progression. Activation of MET via autocrine, paracrine, or mutational mechanisms can lead to tumorigenesis and metastasis, and numerous studies have linked inappropriate expression of this ligand-receptor pair to most types of human solid tumors. Germ line and somatic missense mutations in the *MET* proto-oncogene have been identified in papillary renal cell carcinomas (4, 5), confirming the link between the *MET* gene and cancer. The MET receptor is more frequently activated in sporadic cancers by overexpression or by the presence of an ectopic HGF loop. In addition, HGF/SF has been strongly associated to cancer metastasis by virtue of its ability to promote stromal infiltration and to protect cells from anoikis, that is, from cell death after detachment from basement membrane (for review, see ref 6).

The product of the *HGF* gene is normally produced and secreted by cells of mesenchymal origin, whereas its receptor encoded by the *MET* oncogene is found mainly in epithelial cells, suggesting that this ligand/receptor couple represents a paracrine signaling system for the mesenchymal-epithelial interaction in physiological and pathological conditions (7). Note that the *MET* proto-oncogene was originally identified as a transforming oncogene in a human osteosarcoma cell line (MNNG-HOS), which had acquired tumorigenicity after treatment with a chemical carcinogen (8, 9). We and others showed that MET is neo-expressed in mesenchymal tumors that naturally synthesize and secrete HGF (10–13), suggesting that this autocrine loop may contribute to the pathogenesis of sarcomas. The highest levels of the receptor have been detected in human osteosarcomas (80%) (14), an aggressive tumor that typically permeates the host bone and rapidly expands to the soft tissues. The normal counterpart of the osteosarcoma cell, namely, the osteoblast, does not express the MET receptor.

The *MET* oncogene product is a transmembrane tyrosine kinase (p190^{Met}). The signal transduction pathway activated by MET involves transphosphorylation of the intracellular domain of the MET protein in tyrosine residues located in the so-called multifunctional docking site, which causes concomitant activation of multiple pathways that account for the complex biological responses to HGF stimulation. Efforts have been made to identify the specific signal transducers responsible for the different steps of this process.

Activation of Ras plays a central role in the entire invasive growth process, affecting both cell proliferation and movement. Coupling of the receptor to Ras seems to be necessary and sufficient to trigger cell proliferation through MAPK activation. PI3-kinase is a signal transducer activated in response to HGF either directly by coupling to the receptor or through other transducers/adapters. PKB/AKT, a crucial downstream target of PI3-kinase, is responsible for many of the biological consequences of PI3-kinase activation. The most widely established outcomes of activating the PKB/AKT pathway is the downstream activation of survival genes and genes involved in cell motility in human cancers (58), including bone tumors (59). Impairment of PI3-kinase through specific inhibitors or mutations abrogates several processes involved in metastasis. In fact, mutagenesis of the MET docking site (preventing coupling with PI3-kinase and favoring the binding of Grb2) abolishes the metastatic potential of cells transformed by oncogenic variants of MET (15). In the mirror experiment, mutagenesis of the multifunctional docking site (hyperactivating PI3-kinase and abrogating Grb2 binding) leaves cell scattering and migration unaffected but abolishes transformation. The conclusion is that concomitant activation of both PI3-kinase and Ras is required to elicit invasive growth and metastasis.

In this paper, we have analyzed the effect of the aberrantly expressed MET receptor after HGF stimulation of osteosarcoma cell lines in order to characterize the downstream activated pathways and the phenotypic changes induced by MET activation.

MATERIALS AND METHODS

Cell lines

In this study, we analyzed five cell lines derived from human osteosarcomas. The Saos-2, U-2OS, and MG-63 human osteosarcoma cell lines were purchased from the American Type Culture Collection (Manassas, VA). The IOR/OS9 and IOR/OS10 cell lines were obtained from surgical specimens of high-grade human osteosarcomas at the Istituti Ortopedici Rizzoli (Bologna, Italy) and were previously characterized (12, 16–18). Cell lines were routinely cultured in Iscove's modified Dulbecco's medium (Sigma, St. Louis, MO) supplemented with standard antibiotics and fungizone and 10% inactivated fetal calf serum (FCS) (EuroClone, Wetherby, UK). Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Proliferation assay

To assay mitogenic activity, we seeded cells in 24-well plates (4×10^4 cells/well) and allowed them to attach for 24 h in Iscove's medium supplemented with 10% FCS. The medium was then removed, and the cells were washed twice in phosphate-buffered saline (PBS) and starved in medium devoid of serum and growth factors for 48 h. Cells were then incubated in serum-free medium supplemented with 100 ng/ml HGF with or without 250 nM K252a for 48 h. Treatment with PD98059 was performed by preincubating cells for 2 h. HGF was obtained as a supernatant from baculovirus-infected SF-9 cells as described previously (19). Stimulation with 10% FCS was used as a control. To evaluate cell number, cells were fixed in 2.5% glutaraldehyde in PBS, stained with 0.2% crystal violet in 20% methanol, and solubilized with 10% acetic acid. Cell growth was assessed by measuring absorbance at 595 nm in a Microplate Reader HTS 7000 (Perkin Elmer, Wellesley, MA). ³H-thymidine uptake was measured after adding 2 μCi of [6-³H]thymidine (28.0 Ci/mmol) (Amersham, Piscataway, NJ) in a final volume of 300 μl. Plates were then incubated for 48 h at 37°C in a 5% CO₂ humidified atmosphere. Following incubation, cells were washed with cold PBS and cold 10% TCA, lysed in 0.25 M NaOH. Lysates were placed in Complete LSC-Cocktail (Packard, Perkin Elmer) and CPMs of triplicate samples were determined in a liquid scintillation analyzer (TRI-CARB 2100 TR Packard).

Antibodies and other reagents

The polyclonal antibodies used in this work were obtained as follows: C12, anti-human MET (Santa Cruz Biotechnology, Santa Cruz, CA); 1922, anti-active, and total MAPK (Promega, Madison, WI); anti-active PKB/AKT (Cell Signaling, Beverly, MA); anti-total PKB/AKT (New England Biolabs, Beverly, MA). The MET-specific tyrosine kinase inhibitor K252a, the selective and cell-permeable inhibitor of MAPK kinase (MEK) PD98059, and the specific phosphatidylinositol 3-kinase inhibitors LY294002 and Wortmannin were purchased from Calbiochem-Novabiochem Intl. (San Diego, CA).

Western blotting and immunoprecipitation

For biochemical assays, subconfluent cultures were washed three times with PBS and starved in medium devoid of serum and growth factors for 24 h. Cells were then stimulated with 40 ng/ml HGF for different times. Total cellular proteins were extracted by lysing the cells for 20 min at 4°C with a buffer containing 50 mM Hepes (pH 7.4), 5 mM EDTA, 2 mM EGTA, 10% glycerol, 150 mM NaCl, 1% NP-40 in the presence of protease, and phosphatase inhibitors. Extracts were then clarified at 14,000 rpm for 30 min at 4°C and normalized with the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL) to a protein concentration of 100 µg/ml. Protein extracts were electrophoresed on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels under reducing conditions and transferred onto nitrocellulose membranes (Hybond, Amersham, Piscataway, NJ). Membranes were immunoprobed according to standard methods and nitrocellulose-bound antibodies were detected by the enhanced chemiluminescence (ECL) system (Amersham).

For immunoprecipitation analysis, cells were lysed as described previously. Lysates were incubated at 4°C for 2 h with a protein A-Sepharose solution and polyclonal anti-human MET antibody (Ab). Immunoprecipitates were washed four times with lysis buffer and analyzed by 8% SDS-PAGE.

To assay mitogen-activated protein kinase (MAPK) subcellular translocation, nuclear and cytosolic cell fraction proteins were extracted as follows. Subconfluent cultures were washed twice with cold PBS and scraped in the presence of PBS. After centrifugation, cells were resuspended in 300 µl of ice-cold cell lysis buffer (20 mM Tris/HCl [pH 8], 20 mM NaCl, 0.5% NP-40, 1 mM DTT, 0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin) by gentle pipetting and allowed to swell on ice for 10 min. Samples were then centrifuged (13,000 rpm) for 1 min. Supernatants were saved as the cytosolic fractions. Pellets were resuspended in 50–200 µl of ice-cold buffer C (20 mM Hepes [pH 8], 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin), mixed by pipetting and left on ice for 30 min. The samples were then centrifuged (13,000 rpm) for 1 min. The supernatants were saved as the nuclear fractions and diluted three times with 20 mM Hepes, pH 8.

MAPK and PKB/AKT kinase assays

To assay p44/42 MAPK and AKT kinase activities, nonradioactive IP-kinase assay kits were purchased from Cell Signaling. Cell extracts were prepared according to the manufacturer's protocols. In brief, 200 µg total protein were incubated with resuspended immobilized either phospho-p44/42 MAPK (Thr202/Tyr204) or AKT antibody slurry. After incubation, extracts were centrifuged and pellets were washed twice with lysis buffer (20 mM Tris/HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, 1 mM PMSF) and then washed twice with kinase buffer (25 mM Tris/HCl [pH 7.5], 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na₃VO₄, 10 mM MgCl₂).

For the MAPK assay, pellets were suspended in 50 μ l kinase buffer supplemented with 200 μ M ATP and 2 μ g Elk-1 fusion protein. For the AKT kinase assay, pellets were suspended in 40 μ l kinase buffer supplemented with 200 μ M ATP and 1 μ g GSK-3 fusion protein.

After incubation for 30 min at 30°C, proteins were analyzed by Western blotting, incubating the membrane with phospho-Elk-1 or phospho-GSK-3 primary antibodies and then with HRP-conjugated anti-rabbit secondary antibody. Bound antibodies were revealed with ECL system as described previously.

Matrigel invasion and motility assays

Simulation of cell invasiveness was determined using a modification of the Matrigel method (20). In brief, 10^5 cells were seeded in 6.5-mm plates (Costar, Cambridge, MA) on the upper side of a porous polycarbonate membrane (8.0 μ m pore size polyvinylpyrrolidone-free, Nucleopore, Pleasanton, CA) coated with the artificial basement membrane Matrigel (12.5 μ g per filter; Collaborative Biomedical Products; Becton Dickinson Labware, Waltham, MA). The lower chamber of the Transwell chamber was filled with Dulbecco's modified Eagle's medium (DMEM) containing 2% FCS in the presence of 20 ng/ml of recombinant baculovirus-produced human HGF. After 24 h of incubation, the filters were removed and cells that invaded the Matrigel and attached to the lower chamber of the Transwell were fixed with 11% glutaraldehyde for 15 min at room temperature, washed three times in distilled water, and stained with 0.1% crystal violet in 20% methanol for 20 min. After three washes with water and complete drying, the filters were photographed and cells were counted.

Motility assay was performed using Transwell chambers with polycarbonate filters (8 μ m pore size). HGF in Iscove's medium with or without 10% FCS was placed in the lower compartment of the chamber, and 1×10^5 suspended cells in Iscove's with or without 10% FCS were then seeded in the upper compartments and incubated for 18 h at 37°C. When indicated, the lower compartment contained 250 nM K252a. Cells migrated toward the lower chamber containing HGF, over 16 h, passing through the filter to reach the lower chamber base. Cells adhering to the lower surface were not detected. Migrated cells were counted after fixation and staining as described previously.

Collagen assay

Cells were harvested from culture using trypsin-EDTA and were counted and centrifuged. The cell pellet was then resuspended at a final concentration of 2×10^3 cells/ml in gelling solution prepared essentially as previously demonstrated (21). In brief, eight parts of type I collagen 2 mg/ml stock solution (G Collagen Seromed, Berlin, Germany) was mixed with one part of $10\times$ Iscove's medium and one part of HEPES 0.2 M, pH 7.4, in a sterile tube kept on ice to prevent premature collagen gelation. Cells were resuspended in the cold mixture and aliquots of 0.4 ml were placed into 24-well Costar plates and allowed to gel for \sim 2–3 h at 37°C to allow polymerization before adding 0.15 ml of the collagen solution alone. After a further 2–3 h in the incubator, allowing polymerization of the mixture, 0.4 ml of Iscove's medium plus 10% FCS was added to each well. HGF was added where indicated. Fresh medium was added every 2 days.

RESULTS

Characterization of the active pathways downstream of MET activation in osteosarcoma cell lines

We studied a panel of human osteosarcoma cell lines ([Table 1](#)) previously characterized for the expression and activation of the MET receptor (12). We examined the signaling pathways relevant to cell proliferation and motility activated in these cell lines by HGF. First, we studied the MAPK cascade that is an important transducer of signals from active Ras proteins to the nucleus, where they control multiple transcriptional responses mainly related to the positive modulation of cell growth and proliferation. In mammalian cells, the MAPK kinases, MEK1 and 2 (22–24), phosphorylate ERKs, leading to their activation and (depending on context and duration) nuclear translocation (25). As shown in [Figure 1A](#), HGF stimulation induced ERK1/2 phosphorylation in the following cell lines: U-2OS, Saos-2, IOR/OS9, and IOR/OS10. ERK1/2 phosphorylation at specific residues resulted in its translocation to the nucleus ([Fig. 2A](#)) and enzymatic activity increase ([Fig. 2B](#)), shown by the phosphorylation of its physiological substrate, the Elk-1 transcription factor at Ser 383 (56). Both MAPK and its substrate phosphorylation were rapid, becoming strongly detectable at the earliest time point analyzed (10 min), and then decreasing at subsequent time points. In the other cell line (MG-63), ERKs phosphorylation was barely or not detectable.

We also studied the PI3K/AKT pathway involved in signaling motility and protection from apoptosis mediated by the MET receptor (26–28). Activation of PI3K pathway results in phosphorylation, and consequently activation, of serine/threonine kinase PKB/AKT. AKT activity is regulated both by binding of PI3K-generated lipid products to its pleckstrin homology domain and by phosphorylation. Activation is associated to phosphorylation of the Thr 308 and Ser 473 residues located within the activation loop of its kinase domain and the C terminus, respectively (29). In two cell lines (U-2OS and Saos-2), HGF induced a consistent phosphorylation of PKB/AKT ([Fig. 1B](#)) and consequent PKB/AKT enzymatic activation ([Fig. 2C](#)), shown by the phosphorylation at Ser 21/9, of its physiological substrate GSK-3 α/β (57). A modest AKT response to HGF was also detected in MG-63 and IOR/OS10 cells. AKT phosphorylation was undetectable in the other cell line.

HGF stimulation induces proliferation in MET expressing osteosarcomas

To demonstrate the HGF-dependent proliferative response in osteosarcoma cell lines, we incubated cell cultures with HGF (100 ng/ml). Cell number increase ([Fig. 3](#)) and tritiated thymidine uptake (data not shown) were evaluated after 2 days treatment.

[Figure 3](#) shows that HGF was able to stimulate cell growth in the absence of serum of all but one osteosarcoma cell lines. In MG-63, where the MAPK cascade was not activated by MET, HGF alone is not sufficient to stimulate cell growth, as expected. In cell lines showing HGF-dependent MAPK activation, the addition of 20–40 μ M PD98059, which is a specific MAPK kinase inhibitor, reduced to 80% and 40% HGF-stimulated cell growth, respectively (data not shown). HGF-dependent growth was consistently inhibited by K252a at low concentrations (250 nM), which are active toward tyrosine kinases such as MET and TRK (30) but not PDGF, EGF receptor, or other kinases (31–33). This showed that cell lines lacking MAPK response to HGF

do not proliferate in response to HGF. In U-2OS and IOR/OS9, the cell growth response to HGF occurred in a dose-dependent manner (data not shown).

HGF induces cell motility and invasion of an artificial basement membrane

Cell lines displaying HGF-induced activation of PKB/AKT, namely U-2OS and Saos-2, showed the ability to migrate through the micropore filter of a Transwell chamber in response to a concentration of recombinant HGF of 50 ng/ml ([Table 2](#)). When the assay was performed in the absence of added serum factors, it also allowed the evaluation of the chemotactic effect of HGF in the absence of other chemotactic stimuli. The migration induced by HGF is completely reverted by the presence of the MET inhibitor K252a.

As expected, other serum factors induced a motile response in U-2OS and Saos-2. Both cell lines presented a basal motility that is doubled by serum alone. This response is independent from HGF, because it is not inhibited by K252a ([Table 2](#)).

Pharmacological inhibitors showed that PKB/AKT, but not MAPK, pathway activation plays a central role in motility of these cells. In fact, both serum- and HGF-stimulated motility is significantly inhibited by low concentrations of specific PI3K inhibitors (10 μ M LY294002 or 1 μ M Wortmannin) in the presence of 10% FCS ([Table 3](#)). The specific MAPK kinase inhibitor, PD98059, did not modify cell motility in response to HGF even used at high concentration (50 μ M, data not shown).

HGF also stimulated U-2OS cells, showing the highest PKB/AKT response to HGF, to invade an artificial basement membrane made of collagen, laminin, and glycosaminoglycans (Matrigel). Matrigel chemio-invasion assay is universally thought to be a reliable test to evaluate cancer cell invasiveness (34). This assay elegantly reflects the actual ability of cancer cells to invade and metastasize *in vivo* in response to MET activation (35). In the invasion assay, U-2OS cells show a dose-dependent response to HGF. Cells showed a spontaneous invasiveness but invaded more actively upon stimulation. ([Fig. 4](#)). The dose response to HGF in Matrigel crossing reaches a plateau at a concentration of recombinant HGF of 10 ng/ml ([Fig. 4](#)).

HGF-dependent invasion of the collagen matrix

HGF is a peculiar morphogenetic factor because it is able to induce *in vitro* phenomena recapitulating the “invasive growth” of epithelial cells. These consist of branching and movement of cells embedded in 3D collagen gels. We evaluated whether osteosarcoma cells are also able to invade 3D collagen gels following HGF stimulation. As shown in [Figure 5](#), the presence of HGF alone induces spreading and branching of U-2OS cells starting from HGF concentration of 2 ng/ml. As shown in the figure, U-2OS are unable to spread in a collagen matrix in the absence of HGF also when grown in the presence of 5% fetal bovine serum (control in [Fig. 5](#)). Concentrations over 10 ng/ml HGF dramatically increase the length and number of branches.

DISCUSSION

We and others (12, 36) have previously demonstrated that the *MET* oncogene-encoded receptor for HGF is aberrantly overexpressed in human osteosarcoma, and that overexpression is associated to the progression of bone tumors (14, 37). Here, we show that HGF stimulates the MAPK and the PI3K signal transduction pathways in osteosarcoma cells, leading to proliferation and motility/invasiveness. In response to HGF, osteosarcoma cells proliferate and invade depending on their ability to activate either of the two pathways, respectively.

The contribution of individual growth factors to osteosarcoma onset and progression has been thoroughly investigated. Here, we propose that the input of HGF in osteosarcoma genesis is relevant because a) MET receptors are expressed at high levels in almost 100% of human osteosarcomas; b) HGF is ubiquitous but mainly produced by mesenchymal cells and tumors; and c) mesenchymal cell transformation has been attained with expression of MET receptor in cells secreting the factor or by aberrantly expressing a MET kinase activated by either translocation or mutations (38, 39).

Here, we show that MET activation contributes to osteosarcoma cell proliferation *in vitro*. HGF stimulation of epithelial and nonepithelial cell lines such as skin keratinocytes (40), melanocytes (41), kidney tubular cells (42), and hepatocytes (43) induces cell growth. We have previously studied several cell lines of mesenchymal origin such as osteosarcomas or rhabdomyosarcomas, and we failed to demonstrate a correlation between HGF and proliferation in most cases (unpublished observations). Here, we show that a proliferative response to HGF is associated with cell ability to show MAPK cascade activation by HGF.

Human osteosarcomas show rapid growth *in vivo* and in culture. However, clinical investigations showed that, in high-grade osteosarcomas, proliferation indexes were of no use in evaluating prognosis and in designing therapeutic approaches. Accordingly, P53/MDM-2 proliferation pathways are functionally expressed in osteosarcomas but are not correlated to clinical prognosis of these tumors (44). Other reports showed that the MIB-1 labeling index was correlated only to osteosarcoma grading, in that G3 and G4 tumors revealed higher MIB-1 index than G1 and G2 tumors (45). An obvious explanation of these contradictory data is that the currently available proliferation indexes, which proved to be useful in predicting biological behavior of epithelial tumors, are not indicative of biological behavior of mesenchymal tumors. Furthermore, it is more likely that in high-grade osteosarcoma, the clinical outcome of patients is affected by the metastatic diffusion of tumors. Therefore, the level of tumor vascularization might be a more reliable index of patient prognosis. Indeed more vascularized tumors, such as the teleangiectatic variant, respond to chemotherapy with a complete necrosis, whereas osteosarcomas with a scant vascularity such as the chondroblastic or fibroblastic variants tend to respond to chemotherapy with a lower percentage of necrosis (46).

We have analyzed the properties of a series of cell lines all derived from high-grade osteosarcomas, which commonly also show an invasive-metastatic phenotype. Metastasis and invasion are distinguishing features of osteosarcoma, which is frequently a disseminated disease. Eighty percent of primary osteosarcomas have associated macro- and micrometastases at presentation (47–49). Among them, skip metastases, isolated “satellites,” or microextensions of neoplastic cells in adjacent bone segments are unique to osteosarcoma (50, 51). The latter are

recognized features of this neoplasm, although not defined at the molecular level. Musculoskeletal tumors other than osteosarcomas or rhabdomyosarcomas expressed minor amounts of the MET receptor in a significant percentage of cases (52). These included nonossifying fibromas, osteoblastomas, desmoplastic fibromas of bone, chondroblastomas, and giant cell tumors of bone. More than 50% of all cases scored positive for MET expression and were associated with recurrent or locally aggressive lesions. Sporadic coexpression of the MET/HGF receptor and ligand is also demonstrated. Factors able to mediate cell motility and invasiveness have long been thought to be involved in triggering the progression of osteosarcoma to the most aggressive phenotype.

The MET receptor may be relevant to osteosarcoma progression because its activation by HGF confers functions for metastatic diffusion and local and peripheral invasion of the surrounding tissues. Metastasis is a multistep process. Motility and invasion of basement membrane represent key steps in the process. We found that HGF stimulates osteosarcoma cell motility in the cell lines where MET activation also induced AKT activation. In addition, it is known that MET/HGF receptor induces expression of extracellular proteases and matrix dissolution *in vitro* (39, 53–55). Here, we show that osteosarcoma cells are able to invade collagen matrix in an HGF-dependent way, providing the cells can show AKT response to HGF.

In conclusion, the picture of MAPK and PI3-kinase cooperation emerging from the analysis of different osteosarcoma cell lines representative of different high-grade osteosarcomas, suggests how the HGF/SF and MET receptor couple might function in activating biological properties that might contribute to osteosarcoma progression. The effectiveness of a biological inhibitor such as K252a in modulating the kinase activity and the biological response to HGF *in vitro* might hold therapeutic promise as a drug in the treatment of patients with osteosarcoma.

ACKNOWLEDGMENTS

This research was funded by A.I.R.C. grants to K. Scotlandi, R. Ferracini, and M.F. Di Renzo, and by Italian government grants from Ministero della Salute e Ministero della Istruzione, dell'Università e della Ricerca. N. Coltella and V. Cerisano are recipients of a fellowship from the Italian Foundation for Cancer Research.

We are grateful to Nino Ferraro for skillful computer imaging assistance and to Elaine Wright for editing the manuscript.

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Received August 14, 2002; accepted February 11, 2003.

Table 1**Biological and biochemical features analyzed on the selected osteosarcoma cell lines**

Cell lines	MET	MAPK activ	PKB/AKT activ	Proliferation	Motility
MG-63	+	-	-	-	-
IOR/OS9	+	+	-	+	-
IOR/OS10	+	+	-	+	-
Saos-2	+	+	+	+	+
U-2OS	+	+	+	+	+

Table 2**Motility assay through a Transwell micropore filter with or without HGF (50 ng/ml) and MET kinase inhibitor K252a (50 nM)**

Cell lines	0% FCS	0% FCS + HGF	10% FCS	10% FCS + HGF	10% FCS + HGF + K252a
MG-63	4±2	32±8	10±8	29±11	0
IOR/OS9	17±3	19±7	10±1	22±10	0
IOR/OS10	2±0	3±1	7±2	6±2	4±0
Saos-2	74±8	552±1	146±42	462±9	147±10
U-2OS	29±1	127±7	65±25	139±29	50±10

Table 3**Motility assay through a Transwell micropore filter with or without HGF and PI3K inhibitor LY294002 (10 μ M)**

Cell lines	10% FCS + HGF	10% FCS + HGF + LY294002	10% FCS	10% FCS + LY294002
Saos-2	507 \pm 31	275 \pm 29	374 \pm 44	199 \pm 31
U-2OS	157 \pm 33	87 \pm 14	104 \pm 44	38 \pm 7

Fig. 1

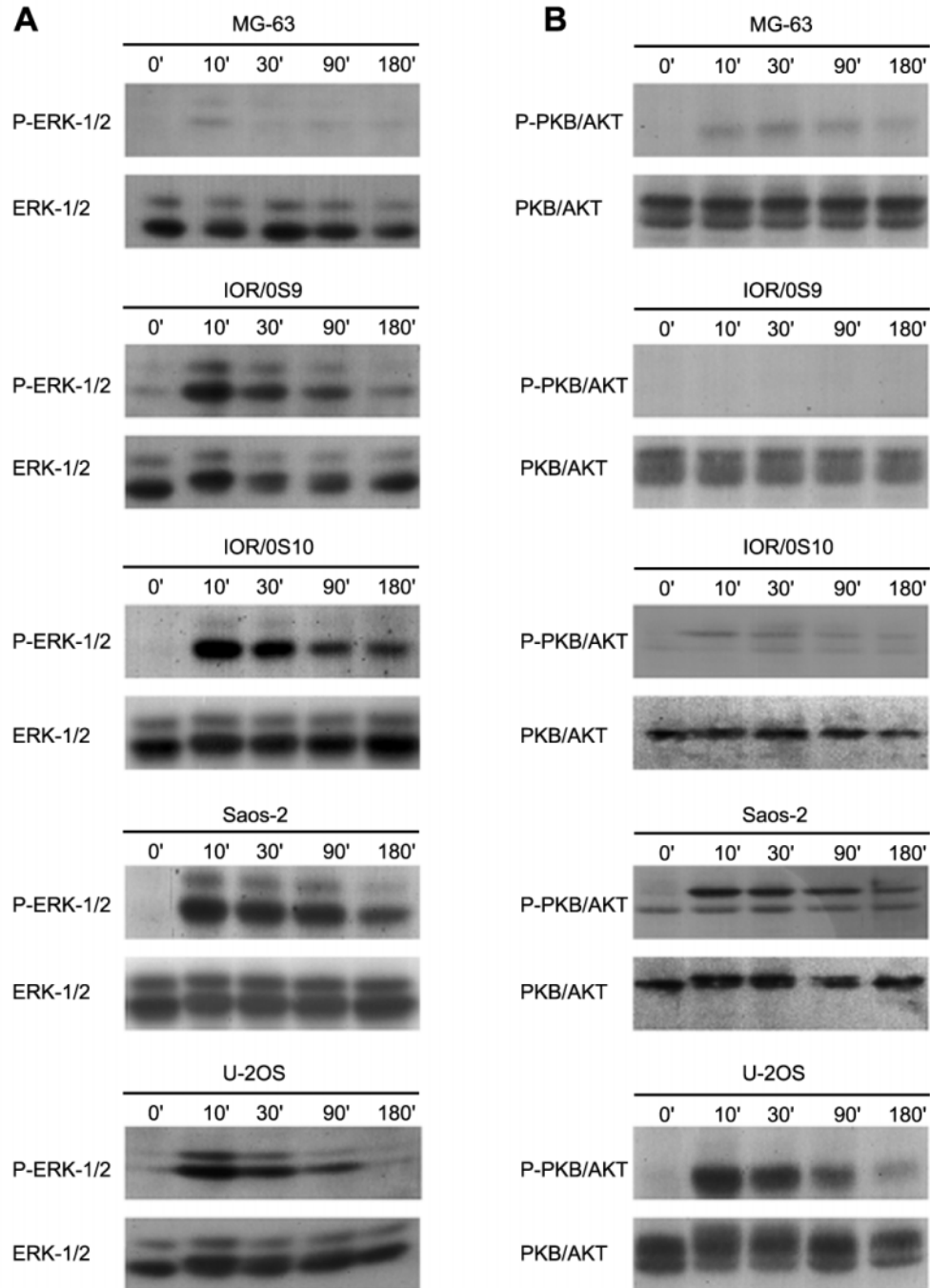


Figure 1. MAPK and PKB/AKT activation by HGF in osteosarcoma cell lines. Osteosarcoma cell lines were starved for 24 h in medium devoid of serum and then stimulated with HGF at 40 ng/ml for the indicated times. Total lysates were resolved by SDS-PAGE, Western blotted, and immunoprobed with antibodies against MAPK (A) or PKB/AKT (B). For each cell line, total proteins were labeled with antibodies directed against either the active form (upper panel) or total protein (lower panel). The active forms of both kinases were identified with antibodies specific for the phosphorylated forms, where phospho-residues are the regulatory ones. In detail, the anti-active MAPK antibody detects the dually phosphorylated, active form of the MAPK enzymes ERK1 and ERK2 as it binds the phosphorylated residues corresponding to the Thr 183 and Tyr 185 of the mammalian ERK2 enzyme. PKB/AKT activation was estimated as phosphorylation in Western blot analysis of total cell extracts with anti-phospho AKT polyclonal antibodies directed against the Ser 473 of AKT1. These antibodies also recognize the equivalent serine residues in AKT2 and AKT3. The presence of equal amounts of AKT protein was estimated by labeling blots with anti-AKT protein antibodies.

Fig. 2

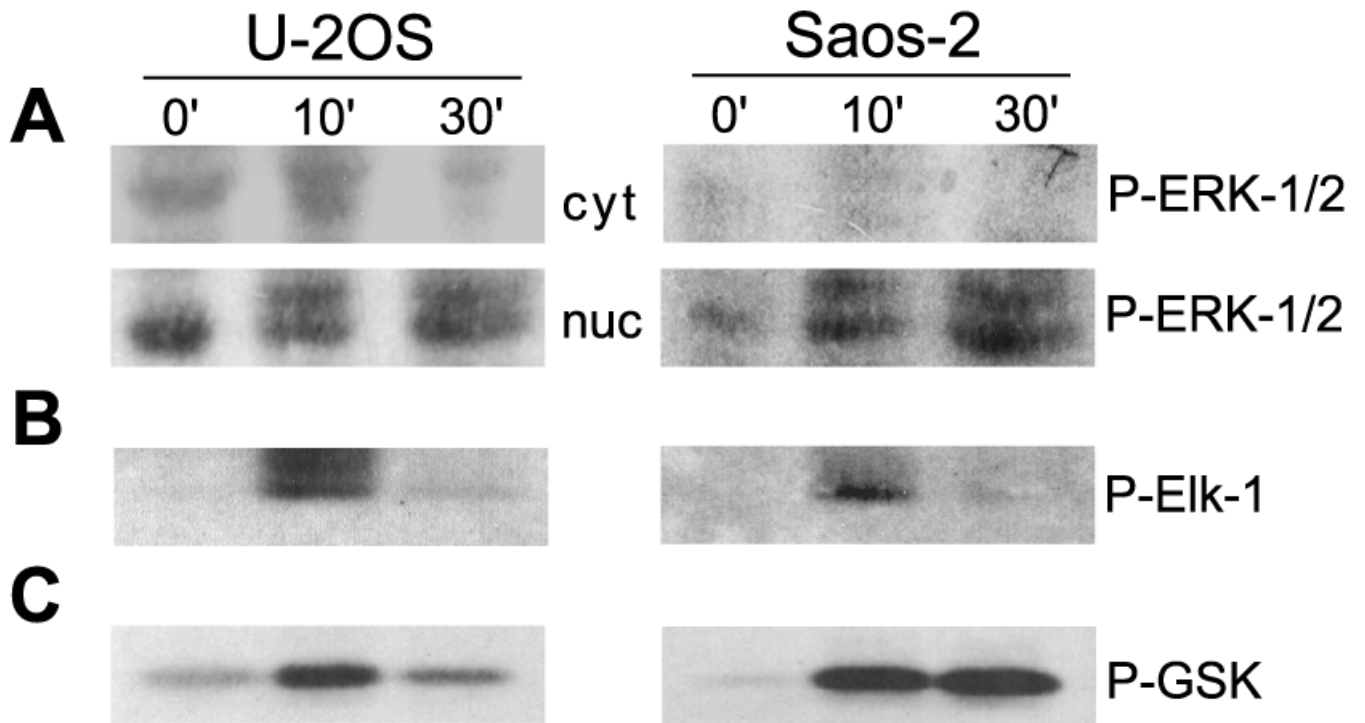


Figure 2. MAPK and PKB/AKT enzymatic activity elicited by HGF in osteosarcoma cell lines. In U-2OS and Saos-2 osteosarcoma cells, MAPK activation by HGF is also shown as both its translocation from the cell cytosol to the nuclear fraction (A) and enzymatic activity toward an exogenously added physiological substrate (B). To reveal MAPK nuclear translocation, cell cytosolic and nuclear fractions were prepared as described in the Materials and Methods. Phospho-MAPK in the two fractions was labeled by specific antibodies (see **Figure 1**) by Western blot analysis. Enzymatic activity of immobilized phospho-MAPK is demonstrated by phosphorylation at Ser 383 of Elk-1, which is revealed with phospho-Elk-1 specific antibodies in Western blot analysis. In the same cell lines, PKB/AKT enzymatic activity triggered by HGF is demonstrated by the phosphorylation of an exogenously added physiological substrate (C). It is the GSK-3 α/β , phosphorylated by PKB/AKT at Ser 21/9 and revealed with phospho-GSK-specific antibodies in Western blot analysis.

Fig. 3

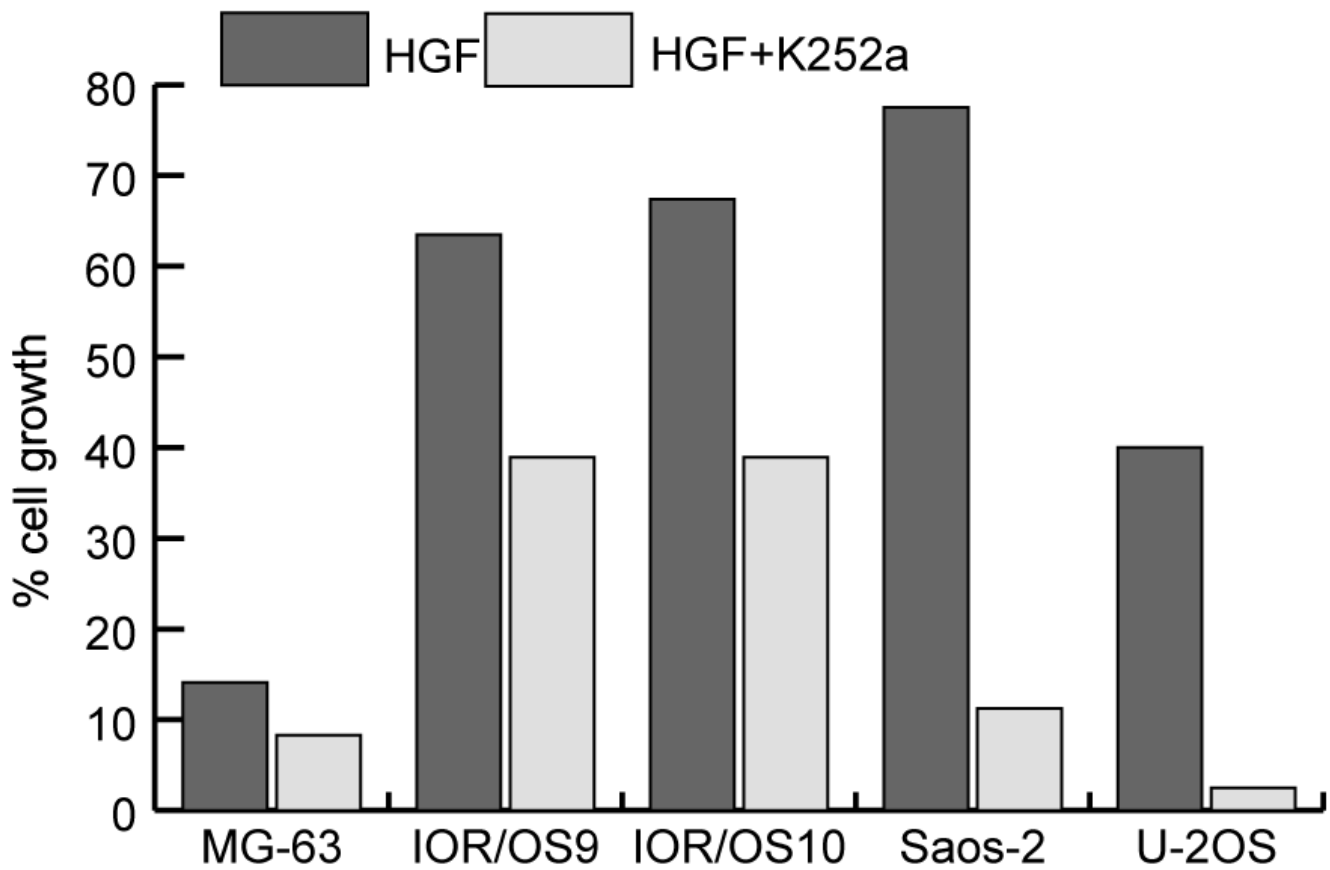


Figure 3. Effect of HGF on osteosarcoma cell proliferation. Cell lines were seeded at low density in 10% FCS for 24 h, starved for 48 h, and then stimulated for a further 48 h with HGF with or without K252a. Cell growth was evaluated by staining cells, solubilizing them, and reading adsorbance at 595 nm in a microplate reader. Numbers on the y-axis of the graph indicate percentage of cell growth compared with a 100% representing number of cells grown in 10% FCS used as positive control.

Fig. 4

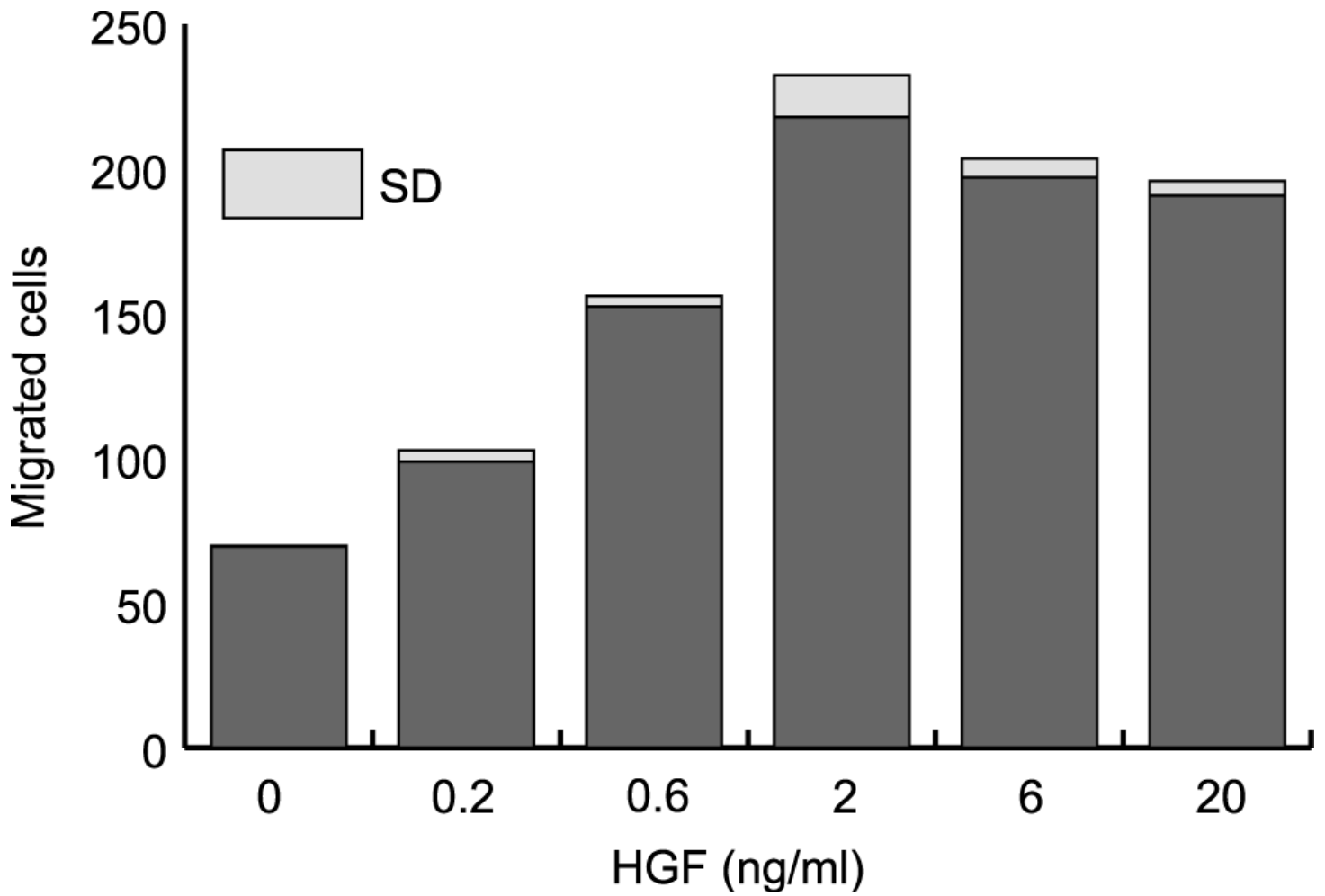


Figure 4. Dose-dependent invasive response to HGF on U-2OS osteosarcoma cell line. The graph shows the effect of HGF in inducing cell invasion through Matrigel, an artificial reconstituted basement membrane. Cells migrating to the lower chamber were stained and counted as described in Materials and Methods. SD, standard deviation.

Fig. 5

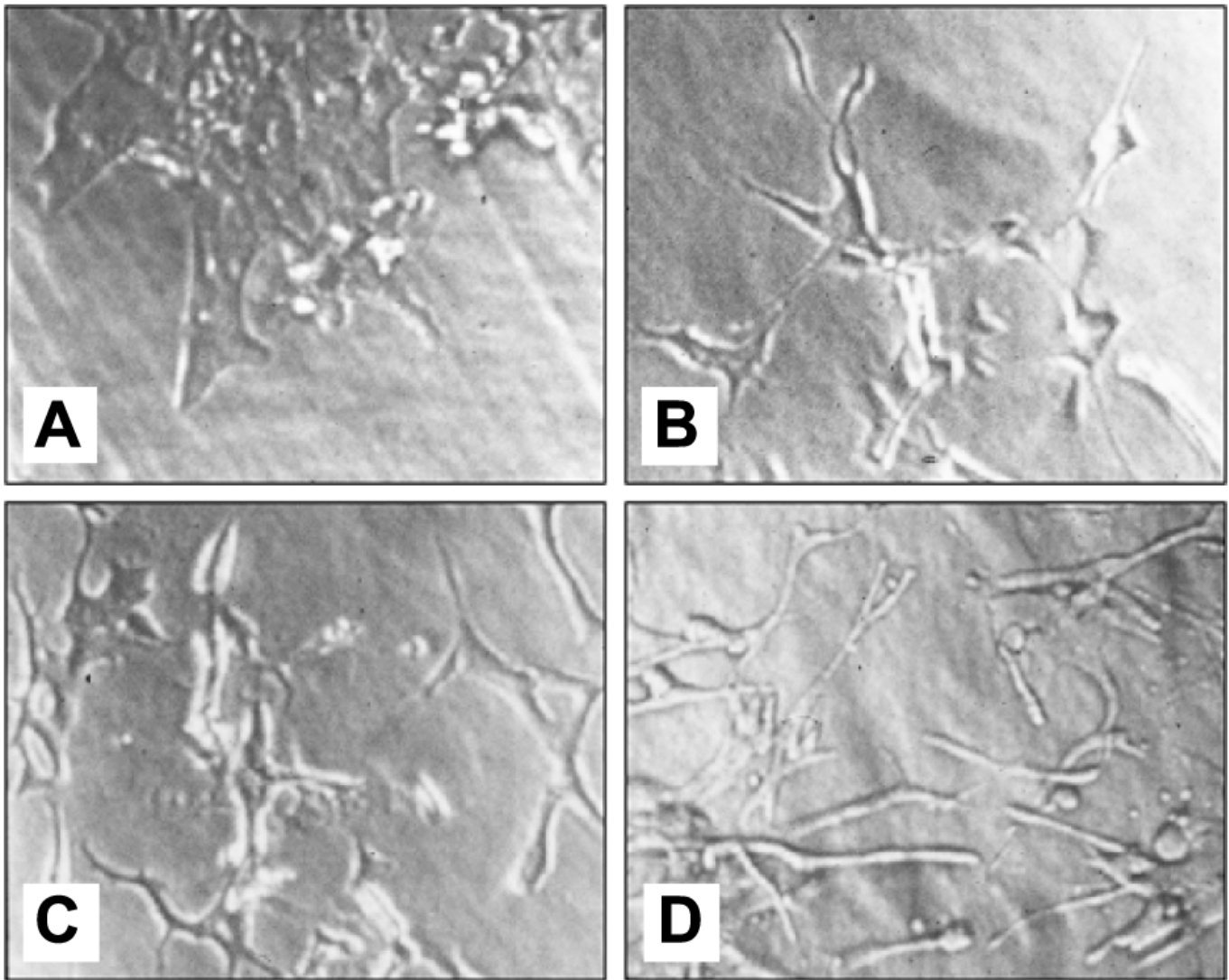


Figure 5. Invasive growth response of U-2OS cells to HGF. Cells grown in collagen gels for 6 days in the presence of 10% FCS were unstimulated (A) or stimulated by adding 2 ng/ml (B), 10 ng/ml (C), or 20 ng/ml (D) of purified HGF. Micrographs of representative fields were taken after 6 days of stimulation.