Mammary Fibroblast-derived Hepatocyte Growth Factor Stimulates Growth and Morphogenesis of Mouse Mammary Tumor Cells in Primary Culture

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We have recently isolated a mammary growth factor from the conditioned medium of mouse mammary stromal fibroblasts and identified it as a mouse homologue of human HGF (hepatocyte growth factor). To elucidate the role of HGF in mouse mammary tumorigenesis, we produced recombinant mouse HGF and examined its effects on primary cultures of mouse mammary tumor cells in this study. HGF at concentrations above 20 ng/ml maximally stimulated the growth of mammary tumor cells in primary monolayer culture. HGF also stimulated the three-dimensional growth and branching morphogenesis of mammary tumor cells cultured inside collagen gels. A comparison of the growth-stimulating activity of HGF with that of EGF (epidermal growth factor) and KGF (keratinocyte growth factor) revealed that HGF is the most potent growth factor among the three. Immunological studies using an antibody against mouse HGF demonstrated that 74% of the growth-stimulating activity present in the mammary fibroblast-conditioned medium was abolished by the antibody, indicating that HGF is the major growth factor produced by the fibroblasts. These observations thus suggest a role for HGF as a mammary stromal fibroblast-derived factor which stimulates growth and morphogenesis of adjacent mammary tumor cells *in vivo*.

Key words: Hepatocyte growth factor — Mammary tumor — Mouse — Collagen gel

It has been frequently observed that epithelial tumor cells isolated from spontaneous mammary tumors of the mouse show only a limited growth potential when placed under *in vitro* monolayer culture conditions.[1] Previously, we hypothesized that the failure of the tumor cells to grow *in vitro* might be the result of separation from the *in vivo* microenvironment surrounding them,[2] since evidence suggested that mesenchymal- (or stromal-)epithelial interactions are important not only for embryonic organogenesis,[3] but also for formation and regeneration of adult organs[4] and for carcinogenesis.[5–7] In an effort to reconstitute the microenvironment which supports the growth and branching morphogenesis of mammary tumor cells we found that mammary stromal fibroblasts produce a potent growth factor.[2, 8, 9] We subsequently identified this factor as a mouse homologue of human hepatocyte growth factor (HGF) through purification, peptide sequencing and cDNA cloning.[10]

The active form of HGF, which consists of a heterodimer of α- and β-chains, was initially purified as a growth factor for primary cultures of liver parenchymal cells.[11–13] The cDNA encoding human HGF was then cloned,[14, 15] and the c-*met* proto-oncogene product, c-Met, was identified as the receptor for HGF.[16, 17] HGF not only stimulates growth,[18] but also promotes scattering,[19, 20] motility,[21] branching morphogenesis of epithelial cells[22] and invasion of cancer cell lines.[23, 24] Cytotoxic activity of HGF on certain tumor cells was also reported.[25, 26] Thus, HGF is now recognized as a multipotent stromal factor

essential for various aspects of stromal- (or mesenchymal-) epithelial interactions.[27]

To elucidate the role of stromal HGF in mammary tumor growth we produced an active heterodimeric form of recombinant mouse HGF in this study and compared its potency with those of other growth factors. The effects of HGF on primary mammary tumor cells were evaluated using two different culture methods, i.e., monolayer culture on plastic and three-dimensional culture inside a collagen gel matrix.

MATERIALS AND METHODS

Animals SHN mice[28] kindly provided by Dr. H. Nagasawa, Faculty of Agriculture, Meiji University, and Dr. T. Mori, Zoological Institute, University of Tokyo, were maintained in our laboratory.
Growth factors EGF (epidermal growth factor, culture grade) was obtained from Becton Dickinson Labware (Bedford, MA). Recombinant human KGF (keratinocyte growth factor) was purchased from GIBCO BRL (Rockville, MD).
Production and purification of recombinant mouse HGF Mouse HGF cDNA[10] cloned in pBluescript II KS vector (Stratagene, La Jolla, CA) was excised and recloned into pBlueBac vector (Invitrogen, San Diego, CA) to yield pBlueBac/HGF. HGF-recombinant baculovirus was prepared using the "MAXBAC" baculovirus expression system (Invitrogen) according to the manufacturer's instructions. Sf9 insect cells cultured in TNM-FH medium (Sigma, St. Louis, MO) were then infected with

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the HGF-recombinant virus. To obtain an active hetero-dimeric form of HGF, 10% fetal bovine serum (FBS, GIBCO BRL) was added to the culture medium.[29–32] HGF was purified from the culture supernatant by a modification of the method of Shimizu et al.[33] using S-Sepharose (Pharmacia LKB, Uppsala, Sweden) and heparin-Sepharose (Pharmacia LKB) with a linear gradient (0.3–1.5 M) of NaCl in 10 mM sodium phosphate buffer (pH 7.4) containing 0.1% CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane-sulfonate). The protein concentration of the HGF preparation was determined by quantification of amino acids after hydrolysis of HGF in a Waters Pico-Tag amino acid analysis system. A portion of the recombinant HGF preparation was analyzed by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.[34]

Immunization and preparation of IgG New Zealand white rabbits were immunized with recombinant mouse HGF (20 μg per rabbit) in complete Freund's adjuvant (Difco, Detroit, MI) and boosted with the same amount of HGF in incomplete Freund's adjuvant. Anti-HGF IgG was purified from the rabbit sera using HiTrap Protein G column (Pharmacia LKB). Preimmune serum obtained prior to immunization was similarly processed to obtain the control IgG.

Assay for growth-stimulating activity Mammary tumor cells were isolated as described previously.[2, 35] Briefly, spontaneous mammary tumors of SHN mice were successively digested with collagenase (Type I, Sigma) and actinase (Kaken Chem. Co., Tokyo), suspended in DME (Dulbecco's modified Eagle's medium; Nissui, Tokyo) supplemented with 10% FBS and 10% dimethylsulfoxide, and stored frozen in liquid nitrogen. For assay, frozen cells were thawed and plated at a density of 10^5 cells per 16-mm well (Microplate, 24-wells; Iwaki Glass, Funabashi) and cultured in 1 ml of DME supplemented with 10% FBS. One day after the plating, various growth factors were added to the wells. DNA content was determined fluorometrically using diaminobenzoic acid (Tokyo Kasei, Tokyo) by the method of Hinegardner[36] as described previously.[2, 9]

Assay for inhibition of growth-stimulating activity Conditioned medium was obtained from the 40th passage of mouse mammary fibroblasts cultured in DME supplemented with 5% calf serum (GIBCO BRL). Nine hundred microliters of DME supplemented with 5% calf serum containing either HGF (final concentration in the medium was 7 ng/ml) or EGF (final concentration in the medium was 21 ng/ml), or 900 μl of the above conditioned medium was preincubated either with anti-HGF IgG or with control IgG for 2 h at 37°C in a 5% CO_2/95% air incubator. The mixture was then added to each well of mammary tumor cells which contained 1 ml of DME supplemented with 10% FBS. DNA content was measured 3 days later.

Collagen gel matrix culture Collagen gels containing 10% FBS were prepared using a type I collagen solution from porcine tendon (Cellmatrix I-A; Nitta Gelatine, Osaka) as described previously.[9, 37] Tumor cells were embedded in collagen gels at a density of 10^5 cells per 16-mm well, overlaid with DME supplemented with 10% FBS and growth factors, and cultured for 7 days. Medium change was done every other day. DNA content was measured after releasing the cells by collagenase treatment as described previously.[9, 37]

Statistical analysis Results are expressed as mean±SE from 3 or 4 wells. Student's t test was used for statistical analysis.

RESULTS

Effect of HGF, EGF and KGF on growth of mammary tumor cells in primary monolayer culture To assess the role of mammary fibroblast-derived growth factor in mammary tumor growth, we first produced the active form of recombinant mouse HGF in a baculovirus expression system. HGF was detectable as a single band of approximately 80 kD under non-reducing conditions, whereas it migrated as two subunits consisting of an α-chain of 68 kD and β-chains of 33–35 kD (Fig. 1). The presence of two β-chains is consistent with the findings reported for human HGF,[38] and may represent two differently glycosylated forms of the β-chain. Using this HGF we examined its growth-stimulating activity on mouse
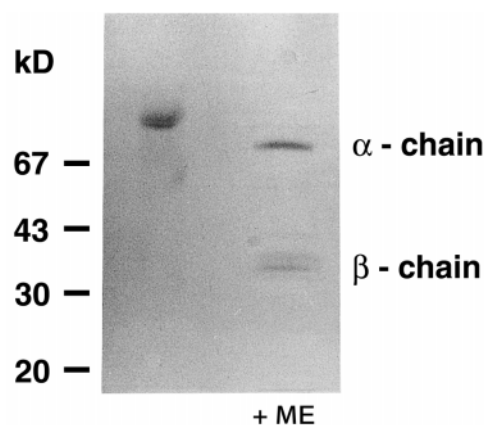

Fig. 1. Electrophoretic analysis of recombinant mouse HGF. HGF was purified from the culture supernatant of HGF-recombinant baculovirus-infected Sf9 cells as described in "Materials and Methods." SDS-polyacrylamide gel electrophoresis was done under non-reducing and reducing (+ME, mercaptoethanol added) conditions using 1.3 μg of HGF in each lane. Proteins were stained with Coomassie brilliant blue. Bars on the left show the locations of molecular weight markers.

mammary tumor cells in primary culture. Tumor cells dissociated from spontaneous mammary tumors of the SHN mouse attached to the substratum and formed a monolayer in a serum-supplemented medium (Fig. 2, A, B and C). In the absence of growth factor supplementation mitotic figures were rare and the tumor cells continued to spread and increase in size (Fig. 2D). When EGF was added to the culture medium, stimulation of growth was observed (Fig. 2E). However, the growth lasted only for a few days. Thereafter, the cells stopped dividing and tended to become enlarged. In contrast to EGF, HGF supported a sustained growth of mammary tumor cells (Fig. 2F). Frequent mitotic figures were present (arrows in Fig. 2F) until the cells reached confluence.

Dose-response curves of growth factors further confirmed the microscopic observations. Supplementation of either HGF or EGF stimulated a dose-dependent growth of mammary tumor cells (Fig. 3A). EGF at a concentration of 20 ng/ml stimulated a 1.9-fold increase in DNA/ well over the control culture in 3 days, while the same concentration of HGF stimulated a 3.8-fold increase. On the other hand, KGF was ineffective in stimulating the growth even at high concentrations. Analysis of the time courses of growth revealed that both the growth rate and the saturation density of the HGF-treated cultures were higher than those of the EGF-treated cultures (Fig. 3B). Thus, mouse HGF proved to be a potent growth factor for mouse mammary tumor cells in primary monolayer culture.
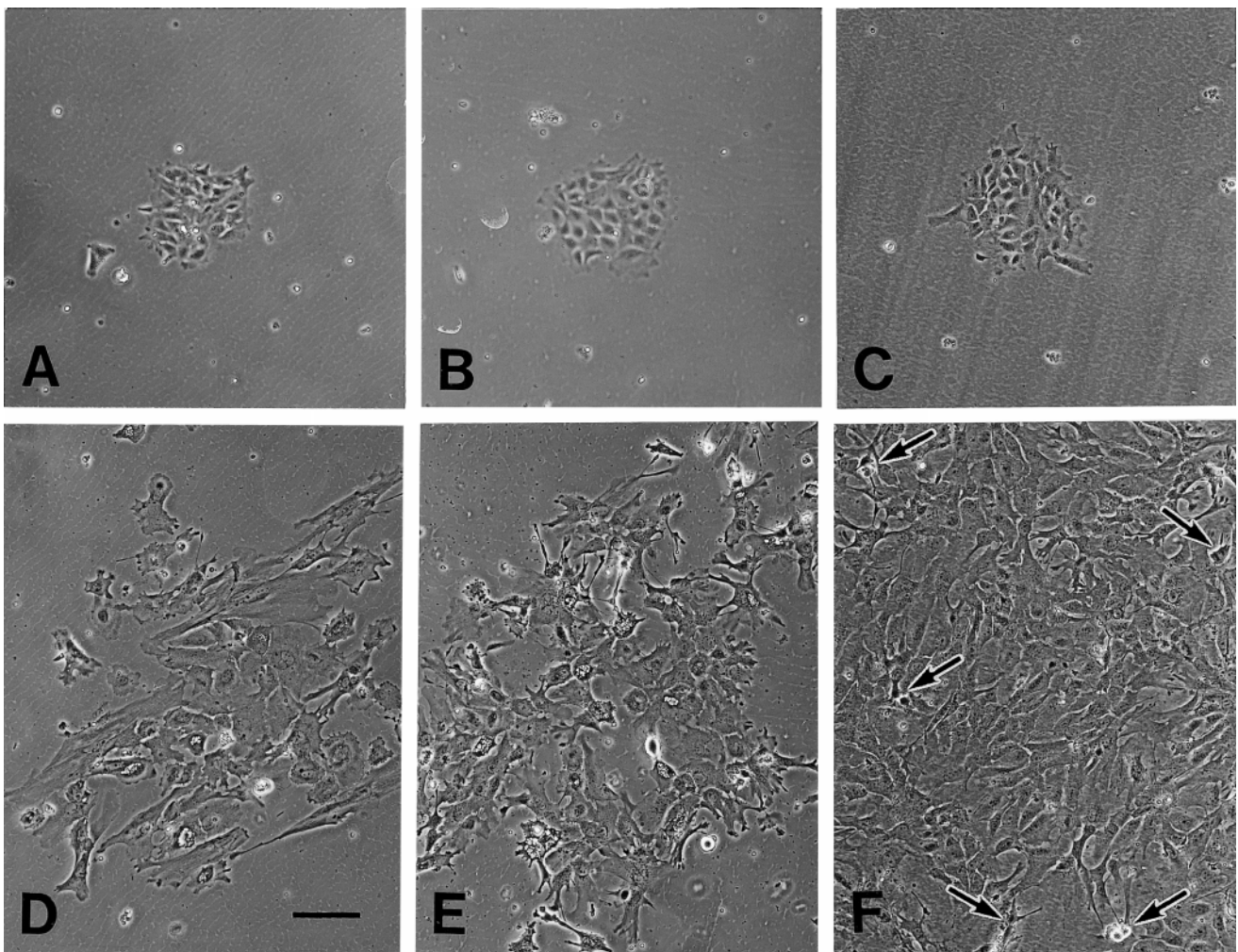

Fig. 2. Photomicrographs of mouse mammary tumor cells in primary monolayer culture. Mammary tumor cells were cultured in DME supplemented with 10% FBS for 24 h (A, B, C). Then, 10 ng/ml of EGF (E) or 10 ng/ml of HGF (F) was added and the culture was continued for a further 68 h. Control culture (D) received no growth factor. A and D, B and E, C and F, are photomicrographs of the same colonies. The horizontal bar indicates 100 μm. Note the presence of mitotic figures in the HGF-treated culture (arrows in F).
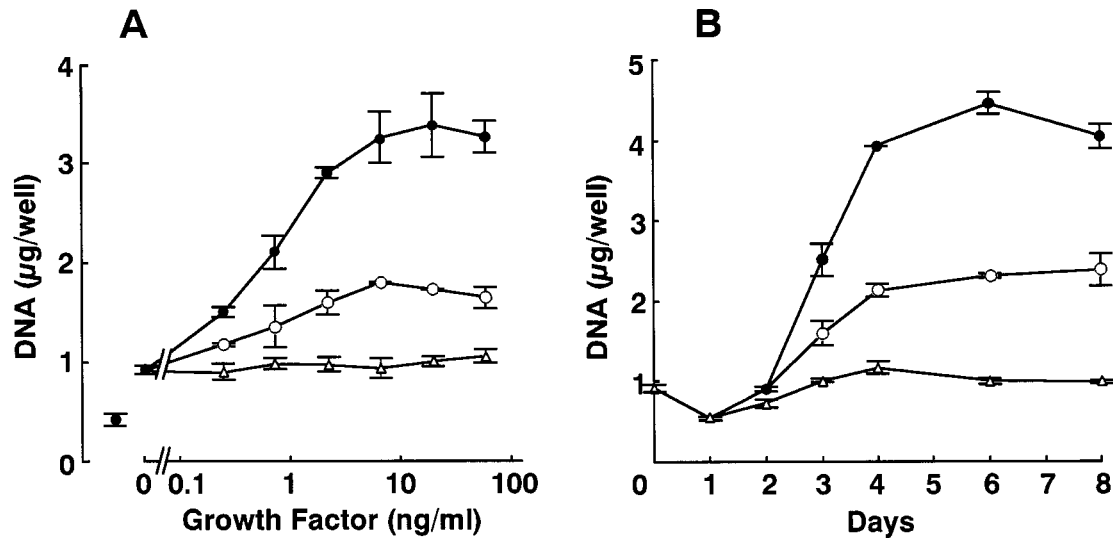
Fig. 3. Effect of HGF, EGF and KGF on growth of mouse mammary tumor cells in primary monolayer culture. Mammary tumor cells were plated in 16-mm wells and cultured in DME supplemented with 10% FBS. (A) Dose-response curves. One day after the plating, various concentrations of HGF (●), EGF (○), or KGF (△) were added to the wells and the culture was continued for 3 days. The leftmost circle represents the DNA content one day after the plating. (B) Time courses of growth. One day after the plating, 20 ng/ml each of HGF (●) or EGF (○) was added to the experimental wells and the culture was continued for a maximum of 8 days. Control culture (△) received no growth factor. DNA content was measured as described in "Materials and Methods." Data are expressed as mean±SE from 3 wells.
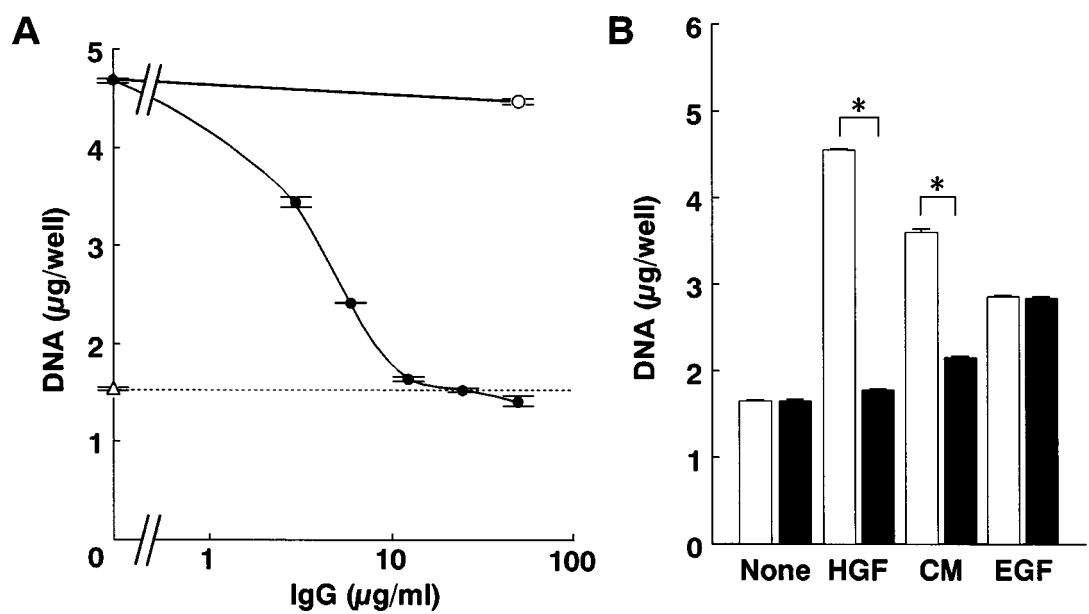

Fig. 4. Effect of anti-HGF IgG on growth-stimulating activities. (A) Inhibition of growth-stimulating activity of recombinant mouse HGF. Mammary tumor cells were cultured in the presence of 7 ng/ml of HGF and various concentrations of anti-HGF IgG (●), 7 ng/ml of HGF and 50 μg/ml of control IgG (○) or in the absence of HGF (△ and dotted line) for 3 days. DNA content was measured. Data are expressed as mean±SE from 3 wells. (B) Inhibition of growth-stimulating activities of mammary fibroblast-conditioned medium (CM) and HGF. Mammary tumor cells were cultured in the presence of 7 ng/ml of HGF, 47% CM or 21 ng/ml of EGF together with anti-HGF IgG (solid columns, 25 μg/ml) or control IgG (open columns, 25 μg/ml) for 3 days. DNA content was then measured. "None" represents cultures maintained in the absence of growth factors or CM. Data are expressed as mean±SE from 4 wells. * P<0.001.
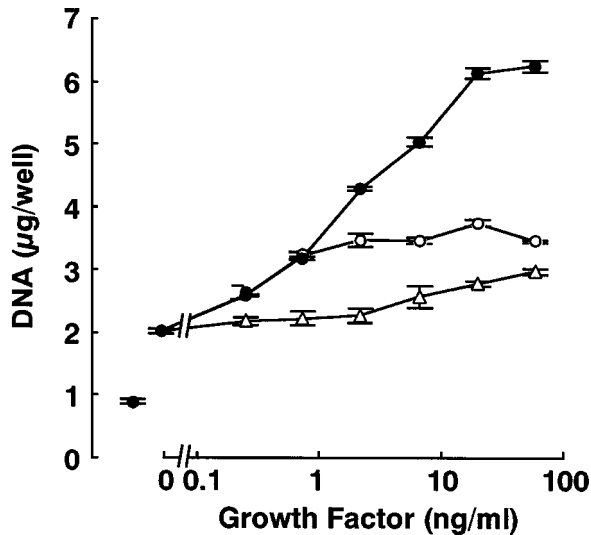
Fig. 5. Effect of HGF, EGF and KGF on growth of mammary tumor cells cultured in collagen gels. Tumor cells were embedded in collagen gels in 16-mm wells and cultured with 1 ml of DME supplemented with 10% FBS and various concentrations of HGF (●), EGF (○) or KGF (△). After 7 days of culture the cells were released from the collagen gels by collagenase digestion and collected by centrifugation. DNA content was then measured. The leftmost circle represents the DNA content at plating. Data are expressed as mean±SE from 4 wells.

Effect of anti-HGF IgG on mammary growth-stimulating activity in the mammary fibroblast-conditioned medium We next examined whether the major growth-stimulating activity present in the mammary fibroblast-conditioned medium is HGF. HGF at a concentration of 7 ng/ml stimulated a 3-fold increase in DNA/well of mammary tumor cells. This stimulatory effect of HGF was inhibited by simultaneous addition of anti-mouse HGF IgG in a dose-dependent manner (Fig. 4A). Control IgG at up to 50 μg/ml failed to inhibit the HGF-stimulated growth. Anti-mouse HGF IgG at a concentration of 25 μg/ml inhibited 96% of the growth-stimulating activity of 7 ng/ml of HGF, while it failed to inhibit the growth-stimulating activity of EGF, showing that the IgG specifically inhibited the growth-stimulating activity of HGF (Fig. 4B). The same concentration of anti-mouse HGF IgG inhibited 74% of the activity in the mammary fibroblast-conditioned medium. These results thus suggest that the major mammary tumor growth factor present in the mammary fibroblast-conditioned medium is HGF.

Effect of HGF on growth of mammary tumor cells cultured in collagen gels Finally, we examined the effect of HGF on growth of mammary tumor cells cultured three-dimensionally in a collagen gel matrix. As in monolayer culture, HGF stimulated the growth of mammary tumor cells in a dose-dependent manner in collagen gel culture (Fig. 5). EGF at a concentration of 20 ng/ml stimulated a 1.9-fold increase in DNA/well over the control culture in 7 days. The same concentration of HGF stimulated a 3-fold increase over the control culture. With regard to the effect of KGF, weak but definite stimulation of growth (a 1.4-fold increase in DNA/well over the control culture) was observed in collagen gels. This positive effect of KGF is in contrast to the absence of its effect in monolayer culture. Photomicrographs of outgrowths formed in collagen gels are shown in Fig. 6. Mammary tumor cells were inoculated as compact aggregates at plating (Fig. 6A). Cultivation in the absence of growth factors for 6 days resulted in the formation of a limited number of branches (Fig. 6B). Both EGF and HGF stimulated growth as well as branching morphogenesis of mammary tumor cells. EGF stimulated the formation of branches thicker than those formed in the HGF-treated culture (Fig. 6C). HGF increased the number of branches, but the branches were thinner than those found in EGF-treated cultures. Thus, outgrowths in the HGF-treated cultures assumed a rather fibroblastic appearance (Fig. 6D).

DISCUSSION

To elucidate the role of stromal fibroblast-derived HGF in growth of mouse mammary tumor cells *in vivo*, we used primary cultures in this study, since the tumor cells are less selected than the cells of established lines and therefore should reflect the *in vivo* characteristics more closely. Mammary tumor cells of the mouse usually lose their growth capacity soon after they are placed under *in vitro* monolayer culture conditions.[1] We have previously hypothesized that the microenvironment surrounding the mammary tumor cells may strongly influence the growth of the tumor cells and it was found that mammary fibroblasts produce a potent factor which stimulates growth and branching morphogenesis of mammary epithelial cells.[2, 8, 9, 39] We subsequently identified this factor as mouse HGF.[10] In this study we obtained an active form of recombinant mouse HGF and showed that HGF alone was sufficient to mimic the effect of mammary fibroblast-conditioned medium on growth and branching morphogenesis of mammary tumor cells in primary culture. We also showed that HGF constitutes the major growth-stimulating activity in the mammary-fibroblast conditioned medium, i.e., it accouts for 74% of the total growth-stimulating activity, and that HGF is more potent than EGF or KGF under monolayer and collagen gel culture conditions. Although KGF was reported to be produced by adult human lung fibroblasts,[40] and purified human KGF stimulates the proliferation of mouse mammary epithelial cells under serum-free conditions,[41] it showed only a limited activity on mouse mammary tumor cells under the
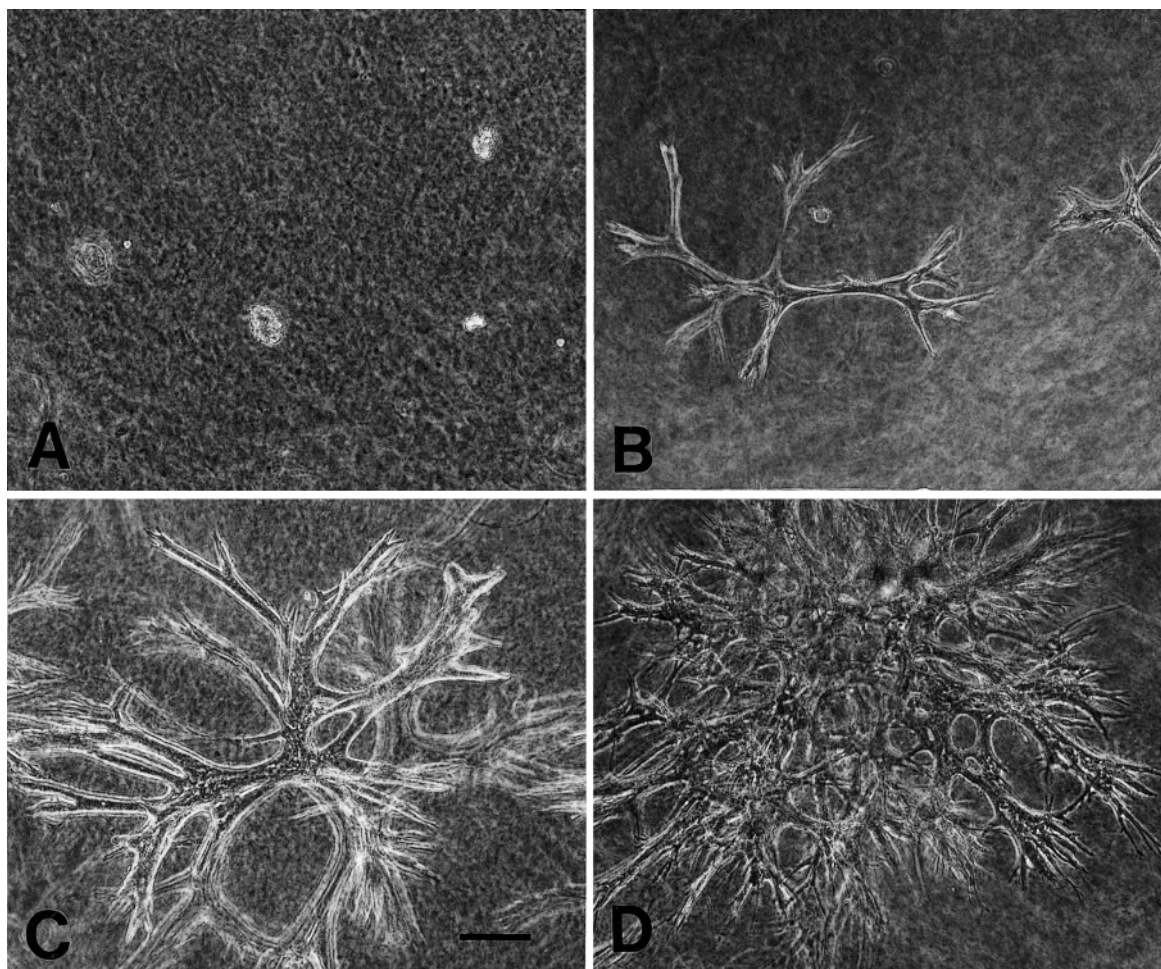
Fig. 6. Effect of HGF and EGF on branching morphogenesis of mouse mammary tumor cells cultured in collagen gels. Phase contrast photomicrographs were taken on the day of plating (A), after 6 days in culture in the absence of growth factors (B), in the presence of 20 ng/ml of EGF (C), or in the presence of 20 ng/ml of HGF (D). The horizontal bar indicates 100 μm.

serum-containing culture conditions employed in this study. As for EGF, a mammary growth-stimulating factor immunologically related to EGF has been reported to be present in the conditioned medium of fetal salivary mesenchymal cells.[42] However, we assume that EGF is virtually absent in the conditioned medium of mammary fibroblasts used in this study, since activity which competes with the binding of ^{125}I-labeled EGF to mammary tumor cells was undetectable in the mammary fibroblast-conditioned medium.[2, 9] It remains to be seen whether the conditioned medium contains other growth factor(s), since the anti-HGF IgG did not completely inhibit the growth-stimulating activity in the conditioned medium in this study.

Mouse mammary tumor cells in primary culture undergo branching morphogenesis in a three-dimensional collagen gel matrix.[43] HGF has been reported to stimulate the branching morphogenesis of mammary epithelial cell lines cultured inside collagen gels.[44, 45] The branching morphogenesis of primary cultures of mammary epithelial cells is also stimulated by HGF as well as by mammary fibroblast-conditioned medium as shown in this study and in our previous studies.[8, 9, 39] In addition, we have shown that HGF as well as mammary fibroblast-conditioned medium stimulated the formation of rather fibroblastic branches of mammary tumor cell outgrowths in contrast to the thick branches stimulated by EGF. The fibroblastic morphology of outgrowths has previously been reported for mammary tumor cells cultured inside collagen gels in the presence of 12-O-tetradecanoylphorbol 13-acetate (TPA), a protein kinase C-activating tumor promoter.[46, 47] TPA induced the disappearance of long actin microfila-

ments prior to the changes in morphology in epithelial cell lines and primary culture of mammary tumor cells in monolayer culture.[48, 49] HGF likewise induced the disappearance of actin microfilaments in addition to reorganization of microtubules and intermediate filaments in epithelial cell lines.[50, 51] In this context it should be mentioned that the HGF-induced cell motility and membrane ruffling are both mimicked by TPA, and that *rho* p21 and *rho* GDI are involved in these processes.[52, 53] Whether the HGF-induced fibroblastic morphology observed in this study is related to these cytoskeletal changes awaits further study.

A variety of fibroblasts including mammary fibroblasts as early as at the secondary passage level and human breast, lung and prostate fibroblasts in primary culture have been shown to produce HGF.[2, 21, 54] HGF produced by the fibroblasts stimulates not only growth, but also scattering, motility and branching morphogenesis of a variety of normal epithelial cells and invasion of tumor cells.[27] These pleiotropic effects of HGF are all transduced by the Met receptor tyrosine kinase[55] expressed on various epithelial cells.[16, 17] Thus, it is conceivable that alteration in the HGF-Met signaling pathway could participate in mammary tumorigenesis and metastasis. Constitutive activation of Met receptor tyrosine kinase by autophosphorylation has been reported in *tpr-met* transgenic mice, and this has resulted in induction of mammary tumors.[56] Constitutive activation of Met by mutation of the c-*met* gene has also been reported.[57] In addition to such Met activation, autocrine or paracrine production of HGF in the Met-positive cells could lead to tumorigenesis. Cell lines having artificially created HGF-Met autocrine loops showed increased tumor incidences in nude mice.[58–60] The presence of such an autocrine loop has recently been identified in a mouse mammary tumor cell line, SP1.[61] The evidence that the HGF transgenic mice developed a wide varietiy of tumors including mammary tumors further suggests the involvement of HGF/Met co-expression and an autocrine loop in mammary tumorigenesis.[62] We have examined the possibility of the presence of an HGF-Met autocrine loop in spontaneous mouse mammary tumors. However, HGF was undetectable in the conditioned medium of the cultured tumor cells. Besides, the tumor cells always required the presence of HGF for sustained growth *in vitro*. Thus, the presence of an HGF-Met autocrine loop or constitutive Met activation has not

yet been identified in spontaneous mammary tumors of the mouse.

In addition to the possible active participation of HGF in mammary tumorigenesis, growth of pre-formed tumor cells could be stimulated by HGF produced by the stroma adjacent to the tumor. Expression of HGF mRNA in the virgin mouse and rat mammary gland has recently been reported from several laboratories.[45, 63–65] We have also confirmed the expression of HGF mRNA in the mouse mammary gland by northern blot analysis (Sasaki, M. and Enami, J., submitted for publication). Moreover, Yang *et al.*[63] demonstrated by *in situ* hybridization that the stromal cells closely surrounding the ductal epithelial cells of the mammary gland express HGF mRNA. Studies led by Sakakura revealed that the fibroblastic mammary mesenchyme which surrounds the mammary rudiment,[5, 66] when transplanted into the adult mammary gland, stimulated the branching morphogenesis of the duct system adjacent to the mesenchyme. In addition, the mesenchyme accelerated the appearance of mammary tumors in mammary tumor virus-carrying mice. Some of the tumors developed directly from the mammary gland adjacent to the transplanted mesenchyme.[67] From these observations, Sakakura[5] suggested that if any alteration occurs in the mammary stroma, such as a change to the embryonic state, the changed stroma would stimulate epithelial growth. A carcinogenic agent, if present at this time, would give rise to cancers with higher frequency. Although the chemical nature of the mediator of the embryonic stromal influence is currently not well understood, HGF derived from the mammary fibroblasts could be one of the candidates. The clarification of this awaits further investigation.

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