Hepatocyte Growth Factor Secreted by Prostate-Derived Stromal Cells Stimulates Growth of Androgen-Independent Human Prostatic Carcinoma Cells

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The objective of the present study is to examine the role of prostate stromal cells on growth and progression of prostate cancer. Co-inoculation of androgen-independent carcinoma cells (PC-3 and CA-7T2) with prostate-derived stromal (P-ST) cells significantly enhanced the growth of carcinoma cells in athymic nude mice. For the *in vitro* study, a three-dimensional co-culture system was used. It consisted of two layers of collagen gel. Stromal cells were suspended in the lower layer, whereas cancer cells were suspended in the upper layer. Compared to the control culture, the presence of P-ST cells in the lower collagen layer significantly stimulated the growth of carcinoma cells. Such an effect was not demonstrated when carcinoma cells were co-cultured with either bone marrow-derived or skin-derived stromal cells. We identified hepatocyte growth factor (HGF) as the principal growth factor released by P-ST cells but not by bone marrow-derived or skin-derived stromal cells. Neutralizing antibodies against HGF completely abrogated the stimulatory effect of P-ST cells. Exogenous HGF likewise stimulated the growth of carcinoma cells *in vitro* and *in vivo*. These results suggest that HGF produced by P-ST cells is a paracrine growth factor that stimulates the growth of androgen-independent prostate cancer cell lines. (*Am J Pathol* 2000, 157:795–803)

Stromal-epithelial cell interaction is important in the maintenance and growth of benign and malignant prostatic epithelial cells. Several laboratories examined the effect of stromal cells on the growth of cancer cells *in vitro* and *in vivo*. In monolayer cultures, the growth of prostate cancer cell lines PC-3 and LNCaP was strongly inhibited by the conditioned medium derived from human prostate stromal cells but not from skin, uterus, and kidney.^{1–3} Co-culture of rat prostatic epithelial cells and prostatic fibroblasts was inhibitory to prostatic epithelial cells unless dihydrotestosterone was added.⁴

In contrast, Camps and colleagues⁵ reported marked stimulatory effects on the growth of cancer cells *in vivo* by stromal cells of variable origins. These stromal cells included a rat prostatic fibroblast cell line, NbF-1; a mouse nontumorigenic fibroblast cell line, 3T3; a mouse mammary fibroblast cell line, C-1271, either irradiated or non-irradiated; a human bone fibroblast cell line, MS, derived from an osteogenic sarcoma; and rat urogenital sinus mesenchymal cells.^{6,7} Despite these extensive studies, it remains unclear whether or not the reported positive influence demonstrated by these prostatic and bone marrow fibroblasts apply to human prostate cancer because of the usage of cells that are clearly abnormal or of nonhuman origin.

The present investigation was performed in an attempt to clarify these seemingly contradictory results between the *in vitro* and *in vivo* studies. We tested the hypothesis that stromal cells of the prostate regulate the growth of androgen-independent prostatic carcinoma cells. We used a three-dimensional co-culture system as an *in vitro* model and athymic nude mice as an *in vivo* model. The former will be an *in vitro* system that would simulate best the *in vivo* growth system. The stromal cells derived from the normal adult prostate, bone marrow, and skin were used. These cells were nontumorigenic as they failed to form tumors in athymic nude mice. Our studies demonstrate that hepatocyte growth factor (HGF) produced by prostate stromal cells is a major growth factor that stimulates the growth of androgen-independent prostate cancer.

Materials and Methods

Cells and Cell Culture

All human tissues used in the present investigation were collected according to the protocol approved by the Institutional Review Board of Northwestern University. We

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used three human prostatic carcinoma cell lines; LNCaP is androgen-sensitive, and PC-3 and CA-7T2 are androgen-insensitive. CA-7T2 is a prostatic carcinoma cell clone established in our laboratory from a radical prostatectomy specimen. A portion of prostate tissue suspicious for carcinoma was incised, and one-half of the sliced tissue was submitted for immediate microscopic examination on cryostat sections. After establishment of the diagnosis of adenocarcinoma (Gleason score, 3 + 3), the remaining half of the tissue was used for primary culture. The tissue was cut into multiple minute cubicles, placed on a plastic surface, and grown in keratinocyte serum-free medium supplemented with 50 μ g/ml bovine pituitary extract, 5 ng/ml epidermal growth factor (EGF), 100 μ g/ml streptomycin, and 100 U/ml penicillin (Life Technologies, Inc., Gaithersburg, MD). As soon as outgrowths formed around the tissue fragments, infection with a retrovirus vector containing the HPV16 E6 gene (LXSN16E6; kindly provided by Dr. Denise Galloway, University of Washington, Seattle, WA) was attempted by the polybrene method. After selection of cells in medium containing Geneticin (G418; 800 μ g/ml; Life Technologies, Inc.), cells were injected subcutaneously (s.c.) in athymic male nude mice. A portion of a tumor that developed after 3 months was returned to primary culture as described above. Cell clones were obtained by the limited-dilution method, and one of the clones, designated as CA-7T2, was used in the present study. CA-7T2 cells expressed neither androgen receptor nor prostate-specific antigen, were androgen-insensitive, and formed an undifferentiated carcinoma in athymic nude mice.

Prostate-derived stromal (P-ST) cells were derived from a cancer-free focus of a prostatectomy specimen removed for cancer. Bone marrow-derived stromal (BM-ST) cells were cultured from the bone marrow of a healthy male donor; heparinized bone marrow aspirates were centrifuged on a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient, and the interface cells were cultured.⁸ Skin-derived stromal (SK-ST) cells were established from the normal abdominal skin of an adult man. All of the cells except CA-7T2 cells were maintained in RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies, Inc.), and incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. For maintenance in the laboratory, CA-7T2 cells were grown in keratinocyte serum-free medium supplemented with 50 μ g/ml bovine pituitary extract and 5 ng/ml EGF (Life Technologies, Inc.).

In Vivo Tumorigenicity Assay

PC-3 cells (5×10^5) or CA-7T2 cells (1×10^6) were suspended in 0.1 ml of serum-free RPMI 1640 medium with or without stromal cells (5×10^5 cells for PC-3 cells and 1×10^6 cells for CA-7T2 cells) and injected at two sites s.c. in the flanks of male athymic nude mice ($n = 6$). In each mouse, the left flank received carcinoma and stromal cells, and the right flank received carcinoma cells alone. In the first experiment, tumors formed were measured weekly, and the assay was terminated at 4 and 6

weeks after inoculation of PC-3 and CA-7T2 cells, respectively. Tumor volumes were calculated with the following formula: volume = length $\times$ width $\times$ height $\times$ 0.5236. Representative portions of all tumors were submitted for routine microscopic examination.

In a second experiment, which was conducted by the identical protocol, CA-7T2 cells were inoculated with or without P-ST cells, and two mice each were killed 48 hours, 96 hours, 1 week, 2 weeks, and 3 weeks later. The injection sites were dissected and submitted for microscopic examination.

In a third experiment, PC-3 cells (5×10^5) were suspended in a mixture of 50 μ l of matrigel (Becton Dickinson Labware, Franklin Lakes, NJ) and 50 μ l of serum-free RPMI 1640 medium with or without recombinant human HGF (50 ng; R&D Systems, Minneapolis, MN) and injected s.c. in the flanks of male athymic nude mice ($n = 6$). Fifty μ l of serum-free RPMI 1640 with or without HGF (50 ng) were inoculated to peritumor sites 2 days, 4 days, and 6 days later. Tumors formed were measured weekly, and the assay was terminated at 6 weeks after inoculation of PC-3 cells. Tumor volumes were calculated as above.

Three-Dimensional Collagen Gel Culture

Collagen gels were prepared as reported previously.⁹ In brief, eight volumes of rat tail type I collagen suspension (Becton Dickinson Labware) were mixed with one volume of 10-fold concentrated RPMI 1640 (Sigma, St. Louis, MO) and one volume of reconstruction buffer (2.2 g of NaHCO₃, 4.77 g of Hepes in 100 ml of 0.05 N NaOH). Collagen gel with or without stromal cells (5×10^4 cells/well) was poured into a 24-well plate (0.5 ml/well; see Figure 2). After incubation for 30 minutes at 37°C to permit complete gelation, a second collagen layer containing carcinoma cells (5×10^4 cells/well) was placed on top of the first layer. After gelation, RPMI 1640 containing 10% FBS was added. FBS was necessary to support the growth of both cancer cells and stromal cells in a 4-day culture. The medium was changed every other day. After incubation for 4 days, each gel layer was removed separately, and carcinoma cells contained in the upper layer were recovered by treatment with 0.1% collagenase I (Worthington Biochemical Corp., Freehold, NJ) and 0.5% trypsin-5.3 mmol/L ethylenediaminetetraacetic acid (Life Technologies, Inc.) and counted with a hemocytometer. A preliminary study indicated that there was no crossover of cells to the adjacent collagen layer. In some experiments, polyclonal rabbit anti-human HGF antibody (20 μ g/ml; R&D Systems), or recombinant human HGF (0 to 100 ng/ml) was added to the above cultures.

Isolation of Cytoplasmic RNA and Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cells grown in monolayers were harvested at early confluency. RNA was prepared by lysing of cells in hypotonic

buffer containing Nonidet P-40 (Sigma), followed by removal of nuclei. Cytoplasmic RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) at 42°C for 60 minutes with use of random primers (5 μ mol/L; Life Technologies, Inc.). Subsequently, 1 μ l of the products was subjected to PCR amplification. PCR was performed as follows. The final concentration of deoxynucleotide triphosphates and primers in the reaction mixture was 200 μ mol/L and 1 μ mol/L, respectively. *Taq* DNA polymerase (Cetus Perkin-Elmer, Norwalk, CT) was added to the mixture at a final concentration of 0.05 unit/ml, and the reaction was carried out in a DNA Thermal Cycler (Cetus Perkin-Elmer). After preliminary testing to determine the optimal PCR condition for semiquantitation, we chose 5 μ g of RNA and 30 cycles for PCR amplification for HGF, keratinocyte growth factor (KGF), and transforming growth factor- β 1 (TGF- β 1); 35 cycles for interleukin-6 (IL-6) and EGF; 22 cycles for vascular endothelial growth factor (VEGF); and 20 cycles of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers were purchased or synthesized according to previous reports: human HGF,¹⁰ KGF,¹¹ IL-6, EGF, and TGF- β 1 (all from Clontech, Palo Alto, CA), VEGF,¹² *c-met*/HGF receptor,¹³ and GAPDH.¹⁴

Collection of Conditioned Medium (CM) from PC-3 and Stromal Cells

Stromal cells were cultured to subconfluency in flasks containing RPMI 1640 medium with 10% FBS. Cells were washed twice with Hanks' balanced salt solution (Life Technologies, Inc.) and downshifted to serum-free RPMI 1640 medium for starvation. After 24 hours, medium was replaced by serum-free medium. Cells were then cultured for an additional 48 hours. The CM was collected and clarified by centrifugation. The protein concentration of CM was adjusted to ~5 μ g/ml by addition of serum-free medium.

PC-3 cells were seeded on a 6-well plate in RPMI 1640 medium and 10% FBS. When they became subconfluent, medium was changed to serum-free medium. After 48 hours, the CM was collected and clarified by centrifugation.

Measurement of Secreted HGF

HGF protein in CM was measured by the Quantikine Human HGF Immunoassay kit (R&D Systems) according to the manufacturer's protocol. CM was collected from collagen gel cultures and monolayer cultures.

Immunohistochemical Demonstration of *c-MET* and HGF Proteins in Athymic Nude Mouse Tumors

Sections were stained for the immunohistochemical expression of cytokeratin (AE1/AE3; DAKO, Carpinteria,

Figure 1. Effect of co-inoculated stromal cells on growth of PC-3 (A) and CA-7T2 (B) cells in athymic nude mice. PC-3 (5×10^5 cells) or CA-7T2 cells (1×10^6 cells) were suspended in 100 μ l of serum-free medium with (dashed line) or without (solid line) stromal cells added 1:1 in ratio and were injected subcutaneously at each flank of male nude mice ($n = 6$). Tumors were measured weekly beginning 1 week (A) or 3 weeks (B) after inoculation, and the experiment was terminated 4 (A) or 6 (B) weeks after inoculation. *, $P < 0.001$; †, $P < 0.01$ compared with respective controls.

CA), vimentin (V9; Oncogene, Cambridge, MA), HGF (AF-294-NA; R&D Systems), and c-Met (C-12; Santa Cruz Biotechnology, Santa Cruz, CA). The avidin-biotin-peroxidase complex method with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used.

Results

Effect of Stromal Cells on Tumor Growth in Vivo

Stromal cells of all sources showed a typical polar spindle shape and showed staining reactions which are indicative of myofibroblastic differentiation. Cells in passages 4 to 8 were used in the subsequent experiments.

We assessed the effect of the presence of stromal cells on the growth of PC-3 and CA-7T2 cells in athymic nude mice. All sites injected with tumor cells developed tumors. Co-inoculation of P-ST cells significantly enhanced the growth of both types of tumor cells, the PC-3 tumors 1.6-fold ($P < 0.01$) and CA-7T2 tumors 4.4-fold ($P < 0.001$). BM-ST cells stimulated PC-3 growth slightly only during the first 2 weeks. SK-ST cells showed no effect (Figure 1A). Both BM-ST and SK-ST cells enhanced the growth of CA-7T2 tumors mildly but significantly (Figure 1B). As compared to PC-3 cells that grow rapidly, CA-7T2 cells were slow in growth and therefore extracellular matrix produced by the stromal cells may have demonstrated growth stimulatory effect better than in fast growing PC-3 cells. By microscopic examination, the tumors were undifferentiated carcinomas with amphophilic cytoplasm, a centrally placed round nucleus, and a prominent nucleolus. Mitoses were frequent. A small amount of connective tissue stroma traversed the mass. The tumors formed after co-inoculation of carcinoma cells and stromal cells were indistinguishable from the tumors formed by carcinoma cells alone.

Figure 2. Effect of stromal cells on growth of PC-3 and CA-7T2 cells in a three-dimensional co-culture system. Collagen containing stromal cells (5×10^4) was poured on the bottom, and a second collagen layer containing PC-3 or CA-7T2 cells (5×10^4) was formed on top of the first layer. After incubation for 4 days, each gel layer was removed mechanically, and cells in the top layer were recovered and counted. Results are expressed as ratio to the respective control culture. Scale bars denote SD of triplicate samples. *, $P < 0.001$ compared with the respective control culture.

Effect of Stromal Cells on Growth of Carcinoma Cells in Three-Dimensional Collagen Gel Culture

We used a three-dimensional co-culture system (Figure 2) with the assumption that it resembled the *in vivo* growth conditions. The presence of P-ST cells in the collagen gel stimulated the growth of PC-3 and CA-7T2 cells 3.2-fold and 1.8-fold, respectively. Such an effect was not demonstrated by either BM-ST or SK-ST cells. The lesser response by CA-7T2 cells may be because of the use of RPMI 1640, which is not an optimal culture medium for these cells (see Materials and Methods). Stromal cells did not increase in number during the culture period (data not shown).

Expression of Growth Factors by Stromal Cells

The above findings suggested that stromal cells secreted growth factors which stimulated the growth of carcinoma cells. Expression of several growth factors was examined by semiquantitative RT-PCR in cells grown on a plastic surface (Table 1). P-ST cells expressed HGF mRNA at a much higher level than did the stromal cells of other types (Figure 3), but VEGF expression was the least among the three cell types. There was no significant difference in the expression of KGF, IL-6, EGF, and TGF- β 1. Expression of HGF mRNA was not detected in any prostatic carcinoma cells (Figure 3).

Table 1. mRNA Expression of Growth Factors by Stromal Cells

Stromal cells	HGF	KGF	IL-6	EGF	TGF- β 1	VEGF
P-ST	+++	+	—	—	+	+
BM-ST	+	++	+	—	+	+++
SK-ST	+	+	—	—	+	+

The results are expressed on an arbitrary scale ranging from — to +++.

Figure 3. Expression of HGF mRNA by semiquantitative RT-PCR. Primers specific for HGF and GAPDH cDNAs generated fragments, respectively, of 691 bp and 782 bp.

We next examined HGF levels in CM by an enzyme-linked immunosorbent assay kit (R&D Systems). Analogous to the expression at the mRNA level, P-ST cells released HGF protein at a high level, and culture conditions did not affect HGF secretion. HGF release by BM-ST, SK-ST, or PC-3 cells was not measurable (Table 2).

Addition of PC-3-derived CM to P-ST cell culture at 50% in a final volume ratio stimulated HGF release by 1.5-fold. Such an effect was not demonstrated by BM-ST or SK-ST cells. When PC-3 and P-ST cells were co-cultured in collagen gel, the HGF level in CM was reduced by ~90% (Table 2). We tested whether this reduction was because of consumption of HGF by PC-3 cells. Exogenous HGF (50 ng/ml) was added to collagen culture in the presence or absence of PC-3 cells. In the culture without cells, HGF was decreased to 22, 18, and 16 ng/ml on days 1, 2, and 3, respectively. On the other hand, in the culture with PC-3 cells, HGF was decreased to 17, 13, and 10 ng/ml on days 1, 2, and 3, respectively. The concentration of HGF was ~40% less in the culture with PC-3 cells. We interpreted the data to indicate that the reduction of HGF in the culture without cells was because of attachment to collagen matrix and that PC-3 indeed consumed a large quantity of HGF.

Expression of *c-met*/HGF Receptor in Prostatic Carcinoma Cells

c-met mRNA was detected in PC-3 and CA-7T2 cells, but not in LNCaP cells (Figure 4). To examine the effect of *c-met* expression on the growth of carcinoma cells, we co-cultured these cells with P-ST cells in collagen gel. The growth of PC-3 and CA-7T2 cells was stimulated significantly in the presence of P-ST cells. In contrast, the growth of LNCaP cells was not affected by P-ST cells (data not shown).

Table 2. HGF Release into CM by PC-3 and Stromal Cells

Cell line and strain	Collagen gel (pg/10 ⁴ cells)	Plastic surface (pg/10 ⁴ cells)
PC-3	nm*	nm
P-ST	1,023 ± 57	1,021 ± 62
BM-ST	nm	nm
SK-ST	nm	nm
PC-3 CM → P-ST	1,534 ± 25	
PC-3 CM → BM-ST	nm	
PC-3 CM → SK-ST	nm	
PC-3 + P-ST	99 ± 28	
PC-3 + BM-ST	nm	
PC-3 + SK-ST	nm	

*Any value under 40 pg/ml is not measurable with the assay kit used. For symbols of cell strains, see footnote in Table 1.

Effect of Exogenous HGF and Anti-HGF Antibody on Growth of Carcinoma Cells in Three-Dimensional Collagen Gel Culture

We next tested the effect of exogenous HGF on the growth of PC-3 cells. A positive response to HGF was observed in a dose-dependent manner (Figure 5). Anti-

Figure 4. Expression of *c-met*/HGF receptor mRNA by prostatic carcinoma cells. Primers specific for *c-met*/HGF receptor and *GAPDH* cDNAs generated fragments, respectively, of 516 bp and 782 bp.

Figure 5. Effect of exogenous HGF on growth of PC-3 cells. Cells (5×10^4) were grown in collagen gel cultures in RPMI 1640 with 10% FBS containing HGF (0 to 100 ng/ml). After incubation for 4 days, cells were counted. Results are expressed as ratio to the respective untreated control culture. Scale bars denote SD of triplicate samples. *, $P < 0.001$; †, $P < 0.01$ compared with the respective untreated control culture.

Figure 6. Effect of anti-human HGF neutralizing antibody on P-ST cell-stimulated growth. PC-3 and CA-7T2 cells (5×10^4) were cultured with or without P-ST cells (5×10^4) in a three-dimensional co-culture system. Anti-human HGF antibody was added to medium at a final concentration of 20 μ g/ml. After incubation for 4 days, cells were counted. Results are expressed as ratio to the respective control culture. Scale bars denote SD of triplicate samples. *, $P < 0.001$ compared with the respective untreated control culture.

HGF neutralizing antibody significantly inhibited the stimulatory effect of P-ST cells (Figure 6).

Sequential Histological Changes at Tumor Cell Inoculation Sites in Athymic Nude Mice

In the *in vivo* tumorigenicity study described above and depicted in Figure 1, co-inoculation of P-ST cells stimulated the growth of cancer cells. *In vitro* assays identified HGF released from stromal cells as a principal growth factor supporting tumor cell growth. However, histological examination failed to demonstrate the presence of spindle cells of presumed prostate origin. To elucidate the role of P-ST cells, we repeated the *in vivo* study. Groups of mice were killed 48 hours, 96 hours, 1 week, 2 weeks, and 3 weeks after inoculation of CA-7T2 cells with or without P-ST cells, and the tissue removed from injection sites was examined microscopically. PC-3 cells were not used in this experiment because they expressed vimentin, which makes identification of cancer cells difficult.

The tissue removed 48 hours after injection of CA-7T2 and P-ST cells demonstrated a sharply demarcated mass (Figure 7,D-F). It showed three distinct histological zones; the center consisted of necrotic cells (designated zone a). It was surrounded by a broad cellular zone (designated as zone b) consisting of spindle cells mixed with degenerated and viable tumor cells. Zone b was surrounded by a rim (designated as zone c) consisting of basophilic viable tumor cells. The anaplastic large cells in zones b and c were positive for cytokeratin (Figure 7E) and reacted positively with the antibody that recognizes human but not mouse c-Met protein (Figure 7M) and were arranged in an anastomosing network. The spindle cells in zone b were confirmed to be P-ST cells because of their positive immunohistochemical reaction to the anti-

Figure 7. Microscopic appearance of injection sites 48 hours, 96 hours, and 1 week after inoculation of CA-7T2 cells (48 hours: **D–F**, **M**, and **N**; 96 hours: **G–I**; 1 week: **J–L**) or without P-ST cells (48 hours: **A–C**). To distinguish cancer cells from stromal cells, we stained sections immunohistochemically for cytokeratin AE1/AE3 (**B**, **E**, **H**, and **K**). To distinguish P-ST cells from stromal cells of host origin, we stained sections with vimentin antibody (**C**, **F**, **I**, and **L**) which recognizes human but not mouse vimentin. Carcinoma cells were diffusely stained positively for cytokeratin and c-Met (**M**), and P-ST cells were reactive with vimentin and HGF antibodies (**N**). **a**, **b**, and **c** refer to zones a, b, and c, respectively.

human vimentin antibody, which does not recognize mouse vimentin (Figure 7F). These cells stained positively with the anti-HGF antibody (Figure 7N).

The tissue removed 48 hours after injection of carcinoma cells alone (Figure 7, **A–C**) revealed a sharply demarcated cystic mass which consisted of a large necrotic zone a and an outer cellular zone c made up of viable carcinoma cells. Zone b of spindle cells was absent.

The mass removed 96 hours after co-inoculation of carcinoma and P-ST cells again showed a three-zone architecture (Figure 7, **G–I**); zone a consisted of necrotic

cells and zone b was predominately made up of P-ST cells with a small quantity of tumor cells arranged in a trabecular pattern. Zone c consisted of tumor cells only.

The mass removed 96 hours after inoculation of carcinoma cells alone was still primarily cystic (zone a) and had a narrow rim of zone c consisting of viable tumor cells. Zone b structure was absent (data not shown). When examined 1 week after co-inoculation of carcinoma and P-ST cells, carcinoma cells continued to proliferate, forming a wide zone c (Figure 7, **J–L**). Spindle cells of P-ST origin were no longer recognizable. Spindle cells

Figure 8. Effect of exogenous HGF on growth of PC-3 cells in athymic nude mice. PC-3 (5×10^5 cells) were suspended in 50 μ l of serum-free medium with (dashed line) or without (solid line) HGF (50 ng) and 50 μ l of Matrigel and were injected subcutaneously at the flank of male nude mice ($n = 6$). Tumors were measured weekly beginning 1 week after inoculation, and the experiment was terminated 6 weeks after inoculation. *, $P < 0.001$; †, $P < 0.01$ compared with the control.

found inside and outside of zone c were unstained by the anti-vimentin antibody, indicating that they were of host origin (Figure 7L).

The mass removed 1 week after injection of carcinoma cells alone was much smaller than the corresponding mass formed after co-inoculation of cells and still exhibited a large cystic center (data not shown). By 2 weeks, the cystic center had been filled with spindle cells of host origin, and the microscopic appearance of tumors was no longer distinguishable between the two groups. P-ST cells could not be recognized (data not shown).

Effect of Exogenous HGF on *in Vivo* Tumor Growth

We examined the effect of exogenous HGF on PC-3 tumor growth. Exogenous HGF was injected to peritumor sites only during the first week in an attempt to recapitulate the effect of short-lived P-ST cells. PC-3 tumor growth was significantly enhanced by treatment with HGF (Figure 8).

Discussion

The present investigation provides evidence to indicate that P-ST cells support the growth of androgen-independent cancers *in vitro* and *in vivo*, and that HGF is one of the major growth factors derived from P-ST cells. HGF synthesis and release were specific for P-ST cells and were not demonstrated by BM-ST or SK-ST cells. Our data suggest that androgen-independent prostatic carcinoma cells (PC-3 and CA-7T2) have an established paracrine support mechanism with P-ST cells that produce HGF. To the best of our knowledge, the present results represent the first report to show that stromal cells of benign human prostate stimulate the growth of human prostate cancer cells *in vivo* and *in vitro*.

HGF is a potent mitogen that was originally isolated from serum because of its ability to stimulate the growth

of hepatocytes *in vitro*.^{15–17} Based on *in vitro* and *in vivo* assays, c-Met/HGF has been proposed as an autocrine/paracrine factor for carcinomas of various organs including the lung,^{18,19} breast,^{20–22} pancreas,^{23,24} stomach,²⁵ gall bladder,²⁶ urinary bladder,²⁷ and prostate.¹³ In the normal prostate gland, c-Met is expressed only in basal cells.²⁸ In carcinomas, c-Met protein expression as detected by immunohistochemistry is variable, but the frequency of c-Met expression increases in cancers of advanced stage.²⁸ These observations suggest possible involvement of c-Met/HGF in androgen-dependent and -independent prostate cancers and, in particular, in advanced cases. Our *in vitro* data provide strong support for this possibility. Furthermore, co-inoculation of P-ST and carcinoma cells enhanced tumor growth *in vivo* significantly. Although enhancement of carcinoma growth by stromal cells was reported previously,^{5–7} these stromal cells were not derived from normal adult prostate. When the injection sites were examined immunohistochemically with anti-HGF and anti-c-Met antibodies, we observed that P-ST cells were positive for HGF and were in close contact with carcinoma cells that expressed c-Met protein (Figure 6, M and N).

The following discussion is offered in an attempt to elucidate the mechanism by which P-ST cells stimulated the growth of cancer cells *in vivo*. Based on the observation that irradiated NbF-1 fibroblasts of rat prostate could support growth of PC-3 cells, Camps et al⁵ suggested that extracellular matrix elaborated by fibroblasts might be able to initiate and/or promote epithelial cell proliferation during tumor formation. This possibility exists in the present study in view of the fact that the growth of CA-7T2 cells (but not of PC-3 cells) was mildly stimulated by co-inoculation of BM-ST and SK-ST cells. It is not likely, however, that the promoting role demonstrated by P-ST cells is merely because of extracellular matrix, because BM-ST cells and SK-ST cells did not show any promoting effect on growth of PC-3 cells. If matrix production were the sole mechanism supporting cancer cell growth, all stromal cells should be equally effective. In their studies, Gleave et al^{6,7} did not observe any effect with two stromal cell lines; one was normal adult lung fibroblast CCD16 and another rat kidney fibroblast NRK.

In the present study, P-ST cells were short-lived, as they were detectable only during the first week, apparently because of the fact that they are nonimmortalized cells. This finding suggests that they are involved in expanding the carcinoma cell population during the early phase of growth by providing HGF.

The cancer cell lines used in the present study are different from clinical androgen-independent prostate carcinomas in that they do not express androgen receptor or secrete prostate-specific antigen.²⁹ Immunohistochemical studies by several groups indicate that androgen receptor is expressed in almost all carcinoma samples including those from metastatic carcinomas. At the same time, however, in all tumor foci immunostaining for androgen receptor is heterogeneous and reveals cells that fail to stain. Furthermore, Gleason score, stage of tumor, or endocrine therapy did not affect the heterogeneity in the staining pattern.^{30–32} Thus, prostate carcino-

mas appear always to consist of a mixture of androgen receptor-positive and -negative cells. The latter type of cells may represent an androgen-independent group of cells as do the cancer cell lines used in the present investigation.

We (unpublished data) as well as others^{10,28} have shown that localized prostate cancer (but not normal prostate columnar) cells express immunohistochemically demonstrable c-Met protein in a high frequency and furthermore that immunohistochemically demonstrable HGF is also frequently expressed in localized and metastatic prostate carcinomas.³³ These findings suggest possible involvement of c-Met/HGF not only in androgen-responsive (-dependent) carcinoma but androgen-independent advanced carcinoma as well. Recently, we have reported that androgen-dependent CWR22 human prostate carcinoma cells respond with significant growth to HGF exposure *in vitro*, and that CWR22R tumor cells derived from CWR22 tumors regrown in male host mice³⁴ after castration produce and secrete HGF.³⁵ Thus, it is quite possible that growth stimulation mediated by c-Met/HGF exists in androgen-dependent and -independent tumors. The fact that *c-met* mRNA is up-regulated in the rat prostate after castration¹⁰ lends support to c-Met/HGF as an alternative or additional mechanism supporting androgen-independent tumor growth.

In conclusion, results of the present study indicate that HGF plays a significant role in the growth of androgen-independent prostate cancer. Our proposed mechanism of HGF action in a paracrine manner may explain the regrowth of androgen-independent prostate cancer at primary site, but may be insufficient to account for the growth at metastatic sites, notably in bone marrow, where HGF release by stromal cells is minimal. In the present study, BM-ST cells did not stimulate the growth of PC-3 carcinoma cells *in vitro* and *in vivo*. HGF derived from the systemic circulation might support the growth of bone marrow metastasis. Alternatively, metastatic prostate carcinoma cells might support themselves by an autocrine mechanism with c-Met/HGF.³³ Additional studies are clearly needed to prove if c-Met/HGF is indeed involved in prostate carcinoma aside from the androgen/androgen receptor system. If c-Met/HGF proved to be involved, c-Met protein can be a target in suppressing the growth of prostate cancer, for example, treatment with a four-kringle antagonist for HGF (NK4).³⁶

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