Aberrant expression of hepatocyte growth factor and its receptor, c-Met, during sex hormone-induced prostatic carcinogenesis in the Noble rat

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Hepatocyte growth factor (HGF) is a multifunctional cytokine which acts as a mitogen, motogen, morphogen and angiogenic factor of epithelial cells. HGF receptor is encoded by a proto-oncogene, c-met, which is overexpressed in various cancers. The role of HGF and c-Met in prostate carcinogenesis, especially in the early stages, is undefined. In this study, prostatic dysplasia and carcinomas were induced by testosterone propionate and 17β-estradiol in Noble rats. The expression of HGF and c-Met was assessed at a protein level by immunohistochemistry and western blot analysis. Intense immunostaining for HGFα and c-Met β-chain was co-localized in dysplastic lesions and in primary and metastatic cancer cells. The levels of HGFα expression were similar among normal control, dysplastic and cancerous prostate tissues, as determined by western blot analysis. Immunoblot study for c-Met under reducing conditions identified two bands at 145 kDa (β-subunit of c-Met) and 170 kDa (precursor form of c-Met) in rat liver extracts. However, two bands at ~220 and 245 kDa were detected in hormone-treated dysplastic prostate tissues and primary tumors. Overexpression of the 220 kDa band was observed in long-term (10–12 months) hormone-treated prostate and primary tumor extracts. Metastatic tumors consistently exhibited up-regulation of a single 245 kDa band. Under non-reducing conditions, however, protein bands of 220, 280 or 300 kDa were seen in the blots. The hormone-treated prostate tissues and metastatic tumors expressed the 220 and 300 kDa proteins, respectively. The majority of primary tumors expressed the 280 kDa protein. In summary, HGF and its receptor, c-Met, were co-expressed in dysplastic and tumor cells, suggesting that an autocrine mode of action may be involved in prostate carcinogenesis. The close correlation of expression of the high-molecular-weight isoforms of c-Met with different stages of carcinogenesis implicates that they might play differential roles in the onset, progression, growth and metastasis in prostate cancer.

Introduction

The c-met proto-oncogene encodes a 190 kDa transmembrane receptor for the hepatocyte growth receptor (HGF) (1,2). The mature receptor (p190met) is composed of a 50 kDa α-subunit and a 145 kDa β-subunit, which are linked by disulfide bonds (3,4), and is synthesized from a single-chain precursor of 170 kDa by terminal glycosylation and proteolytic cleavage (5). HGF is a disulfide-linked heterodimeric glycoprotein of 82 kDa, composed of a 69 kDa α-subunit and a 34 kDa β-subunit (6). The factor is produced mainly by stromal cells and acts predominantly on Met-expressing epithelial cells (7,8).

HGF is a multifunctional cytokine which acts as a mitogen, motogen (i.e. causing cell scattering, migration and extracellular matrix invasion), morphogen (causing cell polarization and tubulogenesis) and angiogenic factor for a variety of normal and neoplastic epithelial cells in vitro (9–12). In vivo, the HGF–Met pair is essential for embryonic development, tissue regeneration, wound healing and cancer invasion (9,13,14).

Aberrant activation of c-Met, as a result of genetic and structural alterations, or changes in the level and pattern of expression, confers tumorigenicity and invasiveness to neoplasms in vitro and in vivo. The c-met protooncogene was originally identified as an oncogene activated by chromosomal rearrangement induced by a chemical carcinogen (15). The resulting hybrid gene product (Tpr–met) has a constitutively active kinase activity, leading to cell transformation (16). Familial inherited and spontaneous mutations in c-met are responsible for hereditary papillary renal cancer (17). The mutationally activated c-Met receptor has been shown to mediate invasive growth and metastasis of cancers (18).

Amplification of the c-met gene is a mechanism of c-Met activation in several types of human cancer (19–21) and in a rodent tumor model (22). Overexpression of the c-Met receptor has been reported in a variety of human malignancies (23–25). Establishment of an autocrine HGF–Met signaling loop in normal cells induces tumorigenesis and the metastatic phenotype (9). In addition, many reports have demonstrated the co-localization of both HGF and c-Met receptors in tumor cells (26–29), suggesting the involvement of autocrine HGF–Met pathway in carcinogenesis. Structural abnormalities of the c-Met receptor due to post-translational processing defects also result in constitutive activation of the downstream signaling pathway (30,31).

HGF is a paracrine mediator of stromal–epithelial interactions in the normal prostate, with stromal cells producing HGF whereas epithelial cells express c-Met receptors (32,33). However, Kurimoto et al. (34) have recently shown the co-expression of HGF and c-Met receptor in human prostate cancer, suggesting the autocrine loop of c-Met activation. Overexpression of the c-Met protein has been observed in malignant prostate tissue (35,36), c-Met is preferentially expressed in androgen-insensitive, metastatic prostate cancer cells such as DU145, PC-3 and MAT-LyLu cell lines (37,38). HGF acts to induce mitosis of normal rat prostate epithelial cells in vitro (32), anchorage-independent growth of human prostate epithelial cells (39) and proliferation, scattering and invasion of DU145 cells (38).

In studying the role of c-Met in prostate cancer, much attention has been put on the late stages of cancer, i.e. invasion and metastasis. To date, however, few studies have addressed...
the role of the HGF–Met signaling system in the onset and progression of prostate cancer. In addition, most data are available from human tumor samples with heterogeneous genetic backgrounds. Recently, we have established an animal model of prostate cancer induced by dual treatment of testosterone propionate (T) and 17β-oestradiol (E2) (40). This animal model not only provides an in vivo system for studying a full spectrum of sex hormone-induced pre-malignant and invasive neoplastic lesions but also minimizes the influence of diversity of genetic factors in specimens. Here, we have demonstrated that HGF and c-Met receptors are co-expressed in dysplastic and tumor cells, suggesting an autocrine activation of c-Met. The two high-molecular-weight isoforms (p220 and p245) of c-Met were identified in dysplastic, primary and metastatic prostate cancer. Overexpression of these isoforms may play a crucial role in the initiation and progression of prostate cancer, in addition to the invasive growth.

Materials and methods

Animals

Male 4–5 week old Noble rats were purchased from Charles River Laboratories (Kingston, NY). The animals were kept under standard conventional conditions (22 ± 2°C, 40–70% relative humidity, 12 h light/12 h dark) and allowed access to food and water ad libitum.

Hormone treatment

Hormone treatments started at 3 months of age, when rats weighted 280–300 g. Silastic tubing (no. 508-009; 1.96 mm i.d.×3.18 mm o.d.; Dow Corning Corp., Midland, MI) of 2 cm and 1 cm length were tightly packed with ~35 mg of testosterone propionate (T) (Sigma, St Louis, MO) and ~25 mg of E2 (Fluka, Buchs, Switzerland), respectively. Both ends of the tubing were sealed with medical adhesive (silicone type A; Dow Corning Corp.) to form the capsules for implantation. Six pieces of 2 cm T capsules and one piece of 1 cm E2 capsule were surgically implanted subcutaneously on the side of the back between the scapula of the rats, under pentobarbitone (Boehringer Ingelheim, Australia) anesthesia. Age-matched control animals were implanted with empty caps. All implants were replaced with new ones every 3 months.

Tissue sampling procedures

Animals were killed by cervical dislocation 0.5, 2, 4, 7, 10 and 12 months after implantation. The entire prostate gland complexes were dissected out from the rats at autopsy. After removing the anterior and ventral lobes, the dorsolateral lobes were bisected along an axis parallel to the prostatic urethra. One-half was rapidly immersed in 10% buffered formalin and were step-sectioned into two or three slabs (in the same plane as the previous bisection), which were then orientated during paraffin embedding such that the face closest to the urethra could be sectioned consecutively toward the periphery of the gland.

For tissue protein extractions, the lateral lobe of the prostate (LP) was excised from the other half of the dorsolateral lobe and further separated into two parts, proximal periurethral and distal glandular regions. Our histological analysis showed that in hormone-treated animals the former region mostly contains ductal carcinoma masses while the latter part mainly contains glandular dysplastic lesions. The separated segments of the LP were snap-frozen in liquid nitrogen and stored at −80°C for subsequent protein extraction and western blotting.

Histopathology

All formalin-fixed samples were routinely processed for light microscopy. Five micrometer thick sections were cut and stained with hematoxylin and eosin for histopathological examination, using the criteria and classification outlined previously (40).

Immunohistochemistry

The goat polyclonal antibody against HGF/T (C-20) and rabbit polyclonal antibody against the Met β-subunit (SP-260) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immunohistochemical staining was performed using the Vectastain Elite avidin–biotin complex (ABC) kit (Vector Laboratory, Burlingame, CA). The sections (5 µm) were dewaxed and then rehydrated in graded alcohol and distilled water. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min, followed by a quick rinse in distilled water and phosphate-buffered saline (PBS). Sections for HGF staining were treated with 0.1% Pronase (Sigma) in PBS for 15 min at room temperature while no antigen retrieval was needed for c-Met immunostaining. Sections were then incubated with normal rabbit or goat serum for 30 min before overnight incubation at 4°C with the primary antiserum against HGF/T (1:150) or Met (1:50). The slides were then incubated with the biotinylated anti-rabbit or anti-goat IgG at a dilution of 1:200 for 30 min at room temperature followed by peroxidase-conjugated avidin–biotin complexes and diaminobenzidine (DAB). The sections were then counterstained with Meyer’s hematoxylin. For controls, the primary antiserum was either preincubated with a 10-fold excess (by weight) of corresponding blocking peptides, HGF/T (sc-1358P) or c-Met (sc-162P) (Santa Cruz Biotechnology), or replaced with the corresponding normal isotype serum (Zymed, San Francisco, CA).

Western blotting

Protein extracts were prepared by homogenizing thawed tissue in a modified radioimmunoprecipitation buffer [50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] containing a cocktail of protease and phosphatase inhibitors (2 mM phenylmethylsulfonylfluoride, 10 µg/ml each of aprotonin, leupeptin and pepstatin A, 1 mM Na3VO4, 50 mM NaF, 5 mM sodium pyrophosphate), using a tissue teard (BioSpin Products, Inc., Bartlesville, OK). The tissue lysate was centrifuged at 10 000 × g for 30 min at 4°C. The supernatant was collected and its protein content was measured using a DC protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of proteins (100 µg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing (5% β-mercaptoethanol or 0.1 M dithiothreitol) or non-reducing conditions. The electrofractionated proteins were transferred to PVDF membranes (Bio-Rad). Blocking and washing steps were performed according to the enhanced chemiluminescence (ECL) instruction manual (Amersham–Pharmacia Biotech UK Ltd, Amersham, UK). The blots were probed for 1–2 h with primary antibodies, anti-HGF/T (C-20) or anti-Met (SP260) antisera under reducing (5% β-mercaptoethanol or 0.1 M dithiothreitol) or non-reducing conditions. The electrofractionated proteins were transferred to PVDF membranes (Bio-Rad). Blocking and washing steps were performed according to the enhanced chemiluminescence (ECL) instruction manual (Amersham–Pharmacia Biotech UK Ltd, Amersham, UK). The immunoreactive signals were detected by ECL western blot detection Corp., Midland, MI) of 2 cm and 1 cm length were tightly packed with reagents (Amersham Biosciences, Uppsala, Sweden). The sections (5 µm) were dewaxed and then rehydrated in graded alcohol and distilled water. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min, followed by a quick rinse in distilled water and phosphate-buffered saline (PBS). Sections for HGF staining were treated with 0.1% Pronase (Sigma)
Table I. Induction of dysplastic lesions and tumors in the LP of Noble rats after T + E2 treatment

<table>
<thead>
<tr>
<th>Months after implantation</th>
<th>No. of T + E2-treated rats</th>
<th>Number (%) of rats with Pre-malignant dysplasia</th>
<th>Microscopic tumors in periurethral ducts</th>
<th>Grossly detectable tumors</th>
<th>Metastasis</th>
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<tbody>
<tr>
<td>0.5</td>
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<tr>
<td>4</td>
<td>9</td>
<td>7 (78)</td>
<td>2 (22)</td>
<td>0 (0)</td>
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</tr>
<tr>
<td>7</td>
<td>11</td>
<td>11 (100)</td>
<td>6 (55)</td>
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<tr>
<td>10</td>
<td>13</td>
<td>13 (100)</td>
<td>12 (92)</td>
<td>1 (8)a</td>
<td>0 (0)</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td>11 (100)</td>
<td>8 (73)</td>
<td>3 (27)b</td>
<td>2 (18)b</td>
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</table>

*After hormone treatment for 10–12 months, there were two animals with gross tumor in the dorsal and lateral lobes of prostate, but no metastasis was observed in distinct organs.

*Macroscopically detectable metastatic lesions were found along the wall of gastrointestinal tract and peritoneum, and within the lung and liver.

(40,41). In this paper, only the points that are directly relevant to the present study will be given.

Normal prostate. The LP, which has been shown to be the most susceptible lobe of prostate to tumor induction by T + E2 (40), consists of epithelium-lined acini and ducts surrounded by a prominent layer of smooth muscle and interspersed fibroblasts (Figure 1).

Prostatic dysplasia. Dysplasia, a pre-malignant lesion, is a disordered pattern of epithelial proliferation within prostatic tubules, characterized by crowding and stratification of epithelial cells with loss of polarization and exhibiting nuclear pleomorphism (Figure 2). In this study, multiple dysplastic foci were scattered in the glandular region of the LP in the treated animals. In the group of animals with short-term (2–4 month) treatment of T + E2, only a small number of epithelial tubules exhibited dysplasia in a given gland, while the majority of tubules remained morphologically unaltered. In contrast, after long-term (10–12 month) hormonal treatment, almost all tubules were involved in variable degrees of dysplastic changes, while morphologically unaltered regions were only found occasionally and focally. Thus, the glandular dysplasia appeared more prevalent in the LP after a much longer period of T + E2 treatment.

Prostate carcinoma. Most of the fully developed prostate carcinomas detected 7 months after treatment were still small (microscopic) tumors that were localized in the periurethral ductal region of the prostate. All these ductal carcinomas were of low grade, i.e. well differentiated (Figure 3), but were usually mingled with focal regions of higher grade, i.e. moderately/poorly differentiated (Figure 4). In a small number of animals with long-term T + E2 treatment (10–12 months), macroscopically gross tumors in the prostate (figure not shown) and distinct metastases to various organs such as, lung, liver, wall of gastrointestinal tract and peritoneum, were also found (Figure 5). Within a metastatic lesion, anaplastic tumor cells aggregated to form isolated islands, chords or trabeculae which were interspersed with surrounding fibroblastic stromal cells (Figure 6).

Immunohistochemistry

HGF: For the normal control prostate, intense immunostaining for HGF was primarily located in the smooth muscle cells; weak staining was occasionally seen in the epithelium (Figure 7a). In the dysplastic region, both epithelial and smooth muscle cells exhibited strong HGF immunoreactivity (Figure 7b). The primary and metastatic tumor cells showed moderate to strong staining for HGF (Figure 7c and d). No apparent correlation of the level of staining to the grade of prostate adenocarcinoma was found. However, very weak staining was observed in the stroma of tumors.

c-Met receptor. c-Met expression was very low or undetectable level in the prostate epithelium and smooth muscles (Figure 8a). Only a few blood vessels demonstrated a moderate level of c-Met expression (Figure 8a, arrow); this endothelium was thus suitable as an internal positive control. Strong and focal immunostaining for c-Met was frequently found in dysplastic epithelial cells (Figure 8b). Heterogeneous immunoreactivities, from weak to strong, were found in primary and metastatic tumors (Figure 8c and d). Likewise, no clear-cut correlation between the staining level for c-Met and the grade of tumors was found.

Negative controls. No immunoreaction was observed in control slides when primary antisera were preadsorbed by the respective blocking peptides or replaced with the corresponding isotype normal sera (Figure 7c and 8c, insets).

Immunoblot analysis

HGF. Immunoblots revealed a band at ~70 kDa consistent with the expected size of the HGF α-chain (Figure 9A). However, densitometric measurement indicated that there was no significant difference in the level of expression among the normal control and hormone-treated prostate and cancer samples (data not shown).

c-Met expression in reducing SDS–PAGE. The antiserum for the β-chain of the c-Met receptor recognized two bands at 145 and 170 kDa in the liver extracts from adult or neonatal rats at the reducing condition (Figure 9B). The 145 and 170 kDa bands correspond to the mature β-chain and the precursor of c-Met, respectively. No band was detected in the normal prostate extracts whereas all the prostate samples from the groups implanted T + E2 either short-term (for 2–4 months) or long-term (for 10–12 months) expressed the 220 kDa band. However, the 245 kDa band was only detected in four (66.7%) of six and one (11%) of nine prostate tissues treated with T + E2 for 2–4 and 10–12 months, respectively. The 220 or 245 kDa proteins, or both of them, were detected in the primary tumor extracts while...
only the 245 kDa band was found in the metastatic tumor samples. Densitometric measurements (Figure 10) revealed that there was a significant increase in the expression of 220 kDa band in the prostate tissues after long-term (10–12 months) T + E\textsubscript{2} implantation, when compared with those after the short-term (2–4 months) treatment ($P < 0.05$). The primary tumor extracts also expressed more of the 220 kDa band than the long-term treatment group. Expression of the 245 kDa protein was significantly (~6-fold) higher in the metastatic prostate tumors than in the primary tumors and hormone-treated prostate tissues ($P < 0.01$).
**c-Met expression in non-reducing SDS–PAGE.** Three protein bands, of 220, 280 and 300 kDa, were seen under non-reducing conditions (Figure 9C). The metastatic tumor extracts only expressed the 300 kDa protein whereas the T + E2-treated prostate extracts only expressed the 220 kDa protein. Heterogeneous expression of all three native protein bands was found in the primary tumor samples, with the majority (75%) expressing the 280 kDa band (Table I).

**Negative controls.** All bands in the blots completely disappeared when the primary antisera were incubated with the corresponding blocking peptides before blotting (Figure 9: panel A, lane 5; panel B, lane 9; panel C, lane 5).

**Discussion**

In the present study, we modified the hormone regimen that was previously established by our laboratory (40,41) by increasing the dosage of testosterone from ~175 mg to ~210 mg, while keeping the amount of estrogen (~25 mg) unchanged. With this modified protocol, our current animal model has achieved what earlier models (40,42–45) failed to do. All animals receiving prolonged treatment (10–12 months) with T + E2 developed not only microscopic prostate tumors, but also grossly visible prostate tumors (organ-confined) and macroscopically detectable metastatic lesions, which are more clinically relevant to human prostate carcinogenesis.

We have demonstrated that, in this system, pre-malignant dysplastic lesions were found scattered in the glandular region of the prostate and appeared more prevalent with longer period of hormonal treatment. However, fully developed tumors (microscopic in nature) were mainly localized in the periurethral area of the prostate, suggesting that the proximal ducts of LP are more susceptible to hormonal carcinogenesis (44). The peculiar feature of the current model in anatomical localizations of pathological lesions enables us easily to isolate the dysplastic tissues (in distal glandular regions) from the cancerous masses (in proximal ductal regions) in hormone-treated LP. Although morphologically unaltered epithelia were found to co-exist with dysplastic lesions even in the LP gland after long-term (10–12 month) hormonal treatment, these apparently ‘normal’ regions displayed distinctive nuclear abnormalities resembling typical dysplasia, as assessed by electron microscopy (unpublished observation). Thus it is believed that almost all the prostatic epithelial cells, particularly after long-term treatment with T + E2, exhibited variable degrees of dysplastic changes. The advantages of the present animal model, should facilitate our study of the underlying mechanisms of prostate carcinogenesis, which is still poorly understood.

The role of HGF and its receptor, c-Met, in metastasis and invasion of prostate cancer has been well established. There is a close correlation between c-Met expression and increased grade of human prostate cancers (37,45). The c-Met receptor is preferentially expressed in androgen-insensitive, highly metastatic prostate cancer cell lines. In vitro studies provided direct evidence that HGF induces proliferation and scattering of prostate cancer cells and increases their invasive potential (37,38). However, the role of HGF and c-Met receptor, other than in invasion and metastasis, in prostate cancer remains poorly defined.

We have demonstrated the co-expression of HGF and c-Met receptor in dysplasia (a pre-malignant lesion), localized and metastatic prostate cancers induced in our model system. Our immunoblot analysis clearly indicated that overexpression of high-molecular-weight isoforms of c-Met was correlated with different stages of prostatic carcinogenesis, suggesting that these aberrant isoforms may play different roles in the development of prostate cancers in the Noble rat.

To evaluate the specificity of the immunohistochemistry and western blot, negative controls were performed by pre-adsorption of primary antisera with an excess of the respective blocking peptide antigens or by replacement of primary antisera with normal sera of the corresponding isotype. Immunostaining in the control slides or bands in the control blots was completely abolished. Since our primary antiserum for the β-chain of c-Met detected bands that differed from the reported size of c-Met β-chain (145 kDa), liver extracts from the adult or neonatal rat were prepared to test the validity of the antibody. These positive control extracts exhibited two bands at 145 kDa and 170 kDa, which corresponded to the β-subunit and the precursor of c-Met, respectively.

In line with previous studies (32,33), immunostaining for HGF was mainly located in the smooth muscle layers of normal prostate. The mild positivity of HGF in the epithelium may be due to the diffusion of this soluble factor from its stromal source. The c-Met protein was barely detectable in the normal prostate by immunohistochemistry and western blot. In normal human prostate, c-Met was found in the basal epithelial cells but not in the luminal epithelial cells. A more sensitive method, using reverse transcription–polymerase chain reaction (RT–PCR), has demonstrated the presence of c-met transcripts in isolated prostate epithelial cells which showed a mitogenic response to the exogenous HGF (32). This observation suggests that c-Met protein is expressed by normal prostate epithelial cells at a very low level. HGF fulfills the requirement as a paracrine mediator in stromal–epithelial interactions, but such interactions may be arrested in the normal adult prostate.

Intense immunostaining for HGF was detected in both epithelial and smooth muscle cells in the dysplastic regions. In primary and metastatic tumors, HGF immunoreactivity was primarily found in the carcinoma cells, but not in the stromal component. Based on this observation, it is likely that the expression of HGF may be switched from the stroma to the epithelium during the course of hormone-induced prostate carcinogenesis. In situ hybridization studies are needed to confirm the site of synthesis of HGF. Co-localization of HGF and c-Met proteins in dysplastic and cancer cells raises the possibility that autocrine mechanism may be exercised in the development of prostate cancers. A previous study (48) has shown that the c-Met protein was frequently detected in dysplastic cells in human prostatic intraepithelial neoplasia; the expression of HGF was not documented. Autocrine HGF–Met signaling has been reported in a variety of human malignancies, including prostate, breast and lung cancers (34,46,47). The induction of sarcoma by co-transfection of HGF and c-met genes into mesenchymal cells (48) supports the autocrine pathway in carcinogenesis.

Although HGF appeared to be ectopically expressed in prostate cancer cells, no apparent increase in the level of HGF expression was found in prostate tumors, as demonstrated in our immunoblot analysis. Tsuka et al. (49) have shown higher expression of HGF protein in non-malignant human prostate samples than prostate cancers. No overexpression of HGF was found in a chemical-induced rat sarcoma displaying c-met gene amplification and overexpression (22). In contrast, over-
expression of HGF has been reported in bladder carcinomas (50).

Our immunoblot studies probed with the c-Met β-chain-specific antiserum showed overexpression of bands at 220 and 245 kDa, also termed p220 and p245, respectively, in the hormone-treated prostate and the induced cancers. At present we can only speculate on the biological significance of these high-molecular-weight β-chain isoforms of c-Met. Expression of the p245 isoform appeared to be correlated with the metastatic capability of tumors. There was a striking increase (~6-fold) in p245 expression in metastatic tumors when compared with the primary tumors. In contrast, the p220 isoform was not detected in metastatic tumor extracts and a high level of p220 expression was observed in primary tumors and prostate tissues after long-term (10–12 months) treatment with hormones. The pattern of expression of p220 and p245 suggests
that these two isoforms may play different roles in the development of prostate cancers. p220 may be more important in the early phase of prostatic carcinogenesis, i.e. initiation and progression, while p245 may play a particular role in the invasive growth of prostate cancers.

The p220 and p245 isoforms recognized in a reducing SDS-polyacrylamide gel differ in molecular size from the reported β-chain of the c-Met receptor (145 kDa). The size difference may be attributed to different post-translational glycosylations, gene mutations or rearrangement, alternative splicing, or defective post-translational processing (15–18,30,31). To investigate whether the p220 and p245 β-chain isoforms would form a heterodimer with the corresponding α-chain, western blots were performed under non-reducing conditions. Three protein bands, of 220, 280 and 300 kDa, were detected. Comparison with the profile of c-Met expression under reducing conditions suggested that the two larger (280 and 300 kDa) proteins appeared to be composed of the aberrant β-chain (p220 or p245) and an α-chain (of ~50 kDa) linked by disulfide bridges. However, a single strong band was detected at 220 kDa under non-reducing conditions in all four prostate tissues with T + E2 treatment and in two (25%) of eight primary prostate cancers, showing that p220 may also exist as a monomeric protein. Overall, our observations raise the possibility that there might be two co-existent species of p220, one forms a 280 kDa complex with other subunits, possibly the α-subunits, while the other does not. It seems that p245 may preferentially bind with other subunits, possibly the α-subunit, forming the dimeric c-Met isoform of ~300 kDa. Further studies are required to characterize the molecular nature of these isoforms and to verify whether they are functional or not.

Multiple mechanisms for generating the aberrant c-Met isoforms may be involved in prostatic carcinogenesis in our model. Different mechanisms have been proposed for the generation of aberrant c-Met receptor isoforms. Defective post-

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**Fig. 7.** Immunohistochemistry for HGFα. (a) Normal control LP. Intense HGFα immunostaining is detected mainly in the smooth muscle cells (arrow). Note that staining in the epithelium is only weak. Magnification: ×180. (b) Dysplasia in the glandular region of hormone-treated LP. Strong immunostaining of HGFα is localized in both dysplastic epithelial cells (arrow) and underlying smooth muscle cells (arrowhead). Magnification: ×360. (c) Carcinoma in the ductal region of the hormone-treated LP. Strong and uniform HGFα reactivity is found in tumor cells. Note the relatively weak reactivity in the thin fibrous stroma permeating this tumor. Magnification: ×360. (d) Carcinoma in the ductal region of hormone-treated LP. Strong and focal immunostaining is found in dysplastic cells (arrows). Magnification: ×180. (e) Carcinoma in the ductal region of hormone-treated LP. Intense and uniform c-Met reactivity is localized in carcinoma cells. Magnification: 360×. No immunoreaction was observed in control slides when the c-Met antibody was neutralized by the corresponding blocking peptide (inset; magnification: ×360). (d) Metastatic tumor. High-level c-Met expression was observed only in metastatic tumor cells, but not in the surrounding fibroblastic stroma. Magnification: ×180.

**Fig. 8.** Immunohistochemistry for the β-chain of c-Met. (a) Normal control LP. Note the very low or undetectable level of the expression of the β-subunit of c-met. Only a few blood vessels show modest staining (arrow). Magnification: ×180. (b) Dysplasia in the glandular region of hormone-treated LP. Strong immunostaining of c-Met is found in both dysplastic epithelial cells (arrows) and underlying smooth muscle cells (arrowhead). Magnification: ×360. (c) Carcinoma in the ductal region of hormone-treated LP. Intense and focal immunostaining is found in dysplastic cells (arrows). Magnification: ×180. (e) Carcinoma in the ductal region of hormone-treated LP. Intense and uniform c-Met reactivity is localized in carcinoma cells. Magnification: 360×. No immunoreaction was observed in control slides when the c-Met antibody was neutralized by the corresponding blocking peptide (inset; magnification: ×360). (d) Metastatic tumor. High-level c-Met expression was observed only in metastatic tumor cells, but not in the surrounding fibroblastic stroma. Magnification: ×180.

**Fig. 9.** Western blot analysis of (A) HGFα under reducing conditions and (B and C) β-chain of c-Met under reducing (B) and non-reducing (C) conditions. Protein extracts (100 μg) were prepared and probed with corresponding antisera, and bands were visualized by ECL. (A) Lane 1, normal control LP; lane 2, LP gland (with prevalent dysplastic lesions) after hormonal treatment for 10–12 months; lane 3, carcinoma in the ductal region of LP after hormonal treatment for 10–12 months; lane 4, metastatic tumor; lane 5, metastatic tumor extracts from lane 4 with pre-incubation of HGFα antiserum with the HGFα blocking peptide. (B) Lane 1, normal control LP; lane 2, LP gland (with only a few dysplastic lesions) after hormone treatment for 2–4 months; lane 3, LP gland (with prevalent dysplastic lesions) after hormone treatment for 10–12 months; lanes 4–6, carcinoma in the ductal region of LP after hormonal treatment for 10–12 months; lane 7, metastatic tumor; lane 8, adult rat liver extract; lane 9, metastatic tumor extract from lane 7 probed with c-Met-specific antibody which was pre-adsorbed with the respective blocking peptide. (C) Lane 1, LP gland after hormone treatment for 10–12 months (a sample from lane 3 of blot B); lanes 2 and 3, carcinoma in the ductal region of LP after hormonal treatment for 10–12 months (samples from lanes 4 and 5 of blot B, respectively); lane 4, metastatic tumor (a sample from lane 7 of blot B); lane 5, metastatic tumor extract, from lane 4 of the same blot, with the c-Met antiserum pre-incubated with the corresponding peptide antigen.

**Fig. 10.** Histogram showing densitometric measurement of p220 (open column) and p245 (hatched column) expression, under reducing conditions, in normal, prostate tissues treated with T + E2 for 2–4 months and 10–12 months, primary prostate tumors and metastatic tumor samples. The ratio at the top of each bar represents the frequency of expression. aSignificantly different from the T + E2 2–4 month treatment group (P < 0.05). bSignificantly different from the T + E2 2–4 month and primary cancer groups (P < 0.01).
translational proteolytic cleavage of the c-Met precursor into mature α and β chains has been reported in colon cancer cells (30) and a gastric cancer cell line (31). This uncleaved c-Met precursor was expressed at the cell surface and constitutively activated. Chromosomal rearrangement, mutations and alternative splicing have been shown to be involved in the aberrant activation of c-Met receptors (15,17,31,51).

In summary, we have demonstrated (i) the co-expression of HGF and its receptor, c-Met, in dysplastic, primary and metastatic prostate cancer cells in vivo; (ii) the overexpression of two isoforms of c-Met β-chain (p220 and p245) during sex hormone-induced carcinogenesis; (iii) the close correlation of expression of p220 and p245 isoforms with different stages of carcinogenesis, suggesting that they may play different roles in onset, progression, growth and metastasis in the prostate cancer. However, the precise role and activation mechanism of these isoforms in prostate carcinogenesis remain to be elucidated.

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References

HGF/Met aberrant expression in rat prostate tumors


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