Activation of Mitogen-Activated Protein Kinase Associated with Prostate Cancer Progression

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Abstract

Using an antibody specific for dually phosphorylated extracellular-regulated kinases 1 and 2, we have examined 82 primary and metastatic prostate tumor specimens for the presence of activated mitogen-activated protein (MAP) kinase. Nonneoplastic prostate tissue showed little or no staining with activated MAP kinase antiserum. In prostate tumors, the level of activated MAP kinase increased with increasing Gleason score and tumor stage. In a separate analysis, tumor samples from two patients showed no activation of MAP kinase before androgen ablation therapy; however, following androgen ablation treatment, high levels of activated MAP kinase were detected in the recurrent tumors. Collectively, these data suggest an increase in the activation of the MAP kinase signal transduction pathway as prostate cancer progresses to a more advanced androgen-independent disease.

Introduction

Early stage prostate cancer typically requires androgen for growth and thus responds to androgen ablation therapy. However, following such therapy, the disease almost invariably progresses to an androgen-independent state, rendering androgen ablation therapy ineffective. This progression of prostate cancer to the frequently fatal androgen-independent disease is associated with the elevation and autocrine production of multiple polypeptide growth factors (1). For example, EGF,7 TGFα, IGF-1, interleukin 6, keratinocyte growth factor, and other FGF family members are expressed in advanced prostate cancers and are believed to be important in fueling androgen-independent growth. Notably, prostate cancer progression is associated with a transition from a paracrine to an autocrine relationship between the EGF receptor and TGFα: in primary prostatic tumors, the neoplastic cells express EGF receptor, and the surrounding stromal cells express TGFα, whereas in advanced disease, the neoplastic cells co-express the EGF receptor and TGFα (2). In addition to an increase in the presence of peptide growth factors, elevated levels of neuropeptides produced by neuroendocrine cells have been associated with prostate cancer progression and decreased patient survival (3, 4). Along with the increased production of neuropeptides and growth factors, increased expression of proliferation markers (e.g., proliferating cell nuclear antigen, KI-67, and MIB-1) correlates with advanced tumor grade and stage in prostate cancer (5).

The growth factors and receptors associated with prostate cancer progression regulate cell growth at least partly through the mediation of Ras family members. These small GTP-binding proteins initiate a signal transduction cascade of successive phosphorylations leading to the activation of various effectors, including MAP kinases (6). Neuropeptides may also influence the activation of MAP kinase by peptide growth factors. A recent study showed that when androgen-responsive LNCaP prostate cancer cells were exposed to suboptimal levels of EGF, elevation of cAMP by neuropeptides dramatically potentiated activation of MAP kinase (7). If signaling by peptide growth factors, such as TGFα, and chronic autocrine stimulation of the Ras pathway play a causal role in prostate cancer progression, one might predict increased activation of downstream kinases, such as MAP kinase, in advanced prostate cancer specimens.

Although few studies have examined the activation of MAP kinase in human tumors, elevated levels of activated MAP kinase have been detected in carcinomas of the kidney, liver, and prostate using electrophoretic mobility shift and immune-complex kinase assays on tumor tissue homogenates (8–10). However, analysis of tumor homogenates may be problematic because nonneoplastic cells are included in the assessment of protein activity. Activation state-specific antibodies have recently emerged as a means to test for activation of specific signal transduction proteins at the cellular level.

In the present study, activation of MAP kinase was assessed using a phospo-MAP kinase antibody specific for the dually phosphorylated and activated MAP kinases ERK1 and ERK2. The results described here show an increase in MAP kinase activation with advanced tumor grade and stage, consistent with the hypothesis that prostate cancer progression is associated with chronic stimulation of the Ras signaling pathway.

Materials and Methods

Tissues.

A total of 82 formalin-fixed (and non-neoplastic tissues were studied. Forty samples were obtained from radical prostatectomies, 23 samples were obtained from transurethral resections (3 transurethral resections are described in the text as part of two case studies), and 19 were metastatic deposits (1 metastasis described as part of a case study). Tumor staging and treatment information for each case was obtained from tumor registry files. Slides were reviewed for Gleason score and stage was assessed as detailed in the American Joint Committee on Cancer, 5th edition (11).

Antibodies.

The antibody specific for the dually phosphorylated MAP kinases, ERK1 and ERK2, has been previously described (12). Briefly, rabbit polyclonal antiserum was raised against the phosphopeptide CHTGFLpEpY-VATR (Quality Controlled Biochemicals, Hopkinton, MA). Affinity purification of antisera was performed by negative selection over a column of nonphosphorylated peptide and subsequent positive selection over a column of dually phosphorylated peptide. Antiserum was used at a concentration of 0.5 μg/ml for immunochemistry. Affinity purified rabbit anti-ERK1/ERK2 (ZS61–7400, Zymed Laboratories, San Francisco, CA), which recognizes both the phosphorylated and nonphosphorylated forms of ERK1 and ERK2, was used at a concentration of 2.5 μg/ml for immunochemistry.
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Immunohistochemistry. Immunohistochemical conditions were optimized using the LNCaP prostate cancer cell line. Serum-starved LNCaP cells were treated with EGF or vehicle for 30 min and fixed in zinc-buffered formalin. To emulsate tissue, LNCaP cells were centrifuged, embedded in agar, and processed routinely into parafin. Under the optimized conditions detailed below, untreated LNCaP cells showed little to no phospho-MAP kinase staining, whereas EGF-treated LNCaP cells showed intense nuclear and cytoplasmic phospho-MAP kinase immunoreactivity (data not shown).

After deparaffinization of unstained human prostate cancer specimens in xylene and alcohol, endogenous peroxidase activity was quenched by 30 min incubation in 0.5% hydrogen peroxide/methanol. Following hydration, microwave epitope retrieval was performed in 10 mM citrate buffer, pH 6.0, for 10 min at 1.15 kW. Peptide competition experiments were performed by preincubating diluted phospho-specific MAP kinase antiserum (0.5 μg/ml) for 1 h at room temperature with a 100 μM concentration of either the dually phosphorylated peptide or the nonphosphorylated peptide. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex method according to the manufacturer’s instructions (Vectastain Elite kit, Vector Laboratories, Burlingame, CA). Biotinylated goat anti-rabbit was used as the secondary antibody and diaminobenzidine was used as the chromogen. Sections were counterstained with hematoxylin.

Digital images for photomicroscopy were acquired with a Dage DC-330 three-color charge-coupled device camera controlled by Image Pro-Plus software. Adjustments in image brightness and contrast were performed identically and in parallel for the images presented using Adobe Photoshop 4.0. Composite images were made using Adobe Illustrator 6.0 and printed on a Codonics NP-1600 dye sublimation printer.

Statistics. A generalized rank test for trend with an assumed known order alternative was used to test, a priori, whether (a) higher levels of activated MAP kinase in samples were associated with higher Gleason scores, and (b) higher levels of activated MAP kinase in samples were associated with more advanced tumor stage. The generalized case III rank test for trend (13) provided the most power to test the particular alternative of interest (i.e., higher levels of activated MAP kinase in tumors with higher Gleason scores).

An exact χ² test was used to test whether levels of activated MAP kinase (zero, low, or high) differed between stage IV primary and metastatic prostate tumors. The power to detect a difference in the range of the observed effect size (0.32) was less than 35%.

Results

Sixty primary prostate tumor specimens were analyzed for activation of MAP kinase: 20 from transurethral resections and 40 from radical prostatectomies. At sampling, only five patients had received prior therapy: four patients received radiation treatment (range, 5 days to 9 years prior to sampling), and one patient had an orchietomy (4 months prior).

Adjacent nonneoplastic prostatic epithelium showed little or no staining with activated MAP kinase antiserum; however, virtually all of these samples displayed some level of activated MAP kinase immunoreactivity in a fraction of the glandular epithelial cells (Fig. 1A). In general, basal cells showed activated MAP kinase staining more frequently than secretory cells, and at times, the basal cell layer showed a nearly continuous staining pattern. Activated MAP kinase was frequently detected in the surrounding smooth muscle stroma, endothelium, and peripheral nerves. Lymphocytes were virtually always negative with only the rare cell staining for activated MAP kinase. In most cells positive for MAP kinase immunoreactivity, both intense nuclear and more diffuse cytoplasmic staining was apparent.

In neoplastic prostatic epithelium, the level of activated MAP kinase varied, ranging from as little as no detectable activated MAP kinase to more than 50% of tumor cells displaying activated MAP kinase. For purposes of statistical analysis, the tumor specimens were grouped into three categories based on the percentage of tumor cell nuclei staining with the phospho-MAP kinase antibody: zero, low (< 10% tumor cells positive), and high (≥ 10% tumor cells positive). This scoring system was highly reproducible and virtually bimodal.

Most samples with low phospho-MAP kinase staining displayed less than 5% positive tumor cell nuclei and most samples with high phospho-MAP kinase staining showed greater than 20% positive tumor cell nuclei. We noted no overt differences in staining in foci of high grade prostate intraepithelial neoplasm compared with adjacent invasive carcinoma. Immunoreactive smooth muscle cells and immunonegative lymphocytes acted as internal positive and negative controls for each sample examined. Fig. 1, B-D, shows three specimens with high levels of activated MAP kinase.

To confirm the specificity of the phospho-MAP kinase antibody, control experiments were performed on a subset of tumors (n = 10). Immunohistochemistry using preimmune affinity purified IgG was utilized (data not shown). Staining with an antibody to total MAP kinase revealed cytoplasmic immunostaining in the majority of epithelial and stromal cells independent of phospho-MAP kinase activation (compare Fig. 2, A and B, to Fig. 2, C and D). In addition, nuclear staining for total MAP kinase was occasionally found in areas of active MAP kinase (Fig. 2D). This subcellular distribution of total and active MAP kinase is consistent with the current understanding of MAP kinase signaling: MAP kinase is activated in the cytoplasm and subsequently translocates to the nucleus. Preincubation of the phospho-MAP kinase antibody with the dually phosphorylated peptide virtually eliminated immunoreactivity (compare Fig. 2, C and E); staining of activated MAP kinase was not blocked when the phospho-MAP kinase antibody was preincubated with the nonphosphorylated peptide (compare Fig. 2, C and F). The ability of the dually phosphorylated peptide to block immunoreactivity was specific to the phospho-MAP kinase antibody, as the phosphorylated peptide was unable to block total MAP kinase staining (data not shown).

Of the 60 primary prostate cancer specimens evaluated, 19 had high levels of activated MAP kinase, 31 had low levels, and 10 had no detectable activated MAP kinase in tumor cells. The relationships between activated MAP kinase, Gleason score, and tumor stage are presented in Tables 1 and 2. The level of activated MAP kinase increased with increasing Gleason score (P < 0.001). High levels of activated MAP kinase were seen in 70% of tumors with a Gleason score of 8–10, whereas low or no activated MAP kinase was seen in 89% of tumors with a Gleason score of ≤6. The levels of activated MAP kinase also increased with increasing tumor stage (P = 0.017); 66% of stage IV tumors had high levels of activated MAP kinase, and 83% of stage II tumors had low or no activated MAP kinase. These data suggest an increase in the activation of the MAP kinase signal transduction pathway as prostate cancer progresses to a more advanced disease.

To further test our hypothesis that elevated levels of activated MAP kinase are associated with advanced disease, we analyzed the level of activated MAP kinase in 18 prostate cancer metastases. Of the 18 metastatic specimens, 15 were from regional lymph nodes, and 3 were from bone or adjacent soft tissue. Prior to sampling, two patients received orchietomies (4 and 16 years previously). In total, 44% (8 of 18) of the metastases showed high levels of activated MAP kinase (7 of 15 lymph node metastases and 1 of 3 bone metastases). No statistically significant difference (P = 0.30) was observed between the levels of activated MAP kinase in metastatic tumor samples compared to stage IV primary tumor samples. Inclusion of the metastatic samples with the primary prostate tumors in an analysis of MAP kinase activation and tumor stage did not change the observed association of increasing MAP kinase levels with advancing stage (P = 0.003).

Our data set of primary and metastatic prostate tumors analyzed above does not enable examination of the relationship between MAP kinase activation and androgen ablation therapy. However, the case histories of two patients not included in the above analyses (because...
we have repeated measures for these patients) suggest that elevated MAP kinase signaling in tumor cells may be selected for during androgen ablation therapy. One patient presented with urinary obstruction due to stage II adenocarcinoma of the prostate (Gleason score, 10). Staining of the TURP done at this time showed no staining for activated MAP kinase. Eleven months later, the patient underwent bilateral orchiectomy, and 7 months following orchiectomy, the patient again presented with urinary obstruction. Analysis of this post-androgen ablation TURP showed high levels of activated MAP kinase. The second patient had an orchiectomy after metastatic deposits were found in his retroperitoneal lymph node dissection. There was no activated MAP kinase detected in this lymph node metastasis. Thirteen years later, the patient underwent a TURP to relieve a urinary obstruction. As with the first patient, this patient’s post-androgen ablation TURP showed high levels of activated MAP kinase. Thus, the data from these patients suggest that activation of the MAP kinase signal transduction pathway and the development of androgen-independent prostate cancer may be related.

Discussion

We examined 82 primary and metastatic prostate tumor specimens for the presence of activated MAP kinase as a measure of Ras signaling using an antibody that specifically recognizes the dually phosphorylated MAP kinases, ERK1 and ERK2. High levels of activated MAP kinase were observed in high-grade and advanced-stage tumors, suggesting elevated Ras signaling in advanced prostate tumors. Although no studies to date have examined the relationship between the level of MAP kinase activation and Gleason score or tumor stage, previous studies have reported elevated expression of MKP-1, ERK1, JNK, and p38 in pre-invasive and invasive prostate cancer (9, 14, 15). However, protein expression is not equivalent to protein activity. For instance, analysis of erbB-2 activity in breast tumors using phospho-specific antibodies suggests variability in erbB-2 activation relative to protein expression levels (16). The above studies assessing the expression of MKP-1, ERK1, JNK, and p38 in prostate tumors also examined the activation of ERK1 and JNK in a subset of tumors using immune-complex kinase assays of tissue homogenates. Both ERK1 and JNK were elevated in some of the tumors examined, whereas JNK activity inversely correlated with MKP-1 expression. However, analysis of protein activity using tissue homogenates is complicated by the presence of nonneoplastic tissue. Furthermore, our observation of activated MAP kinase in nonneoplastic prostate tissue underscores the difficulty in interpreting studies using tissue homogenates.

We have used the emerging technology of activation state-specific antibodies to directly examine activation of the MAP kinase signaling
pathway in prostate cancer. The activation state of ERK1 and ERK2 was assessed at the cellular level by using an antibody that specifically recognizes dually phosphorylated ERK1 and ERK2. A similar approach was recently used to demonstrate activation of MAP kinase in a broad range of glial tumors (astrocytic tumors, glioblastomas, and oligodendroglialomas) (17). Notably, oligodendroglialomas showed an increase in MAP kinase activation with malignant progression.

Previous studies have demonstrated a role for MAP kinase activa-

Fig. 2. Specificity of staining for activated MAP kinase in a prostate tumor. Serial sections of the same neoplasm were stained. A and C, phospho-MAP kinase staining; B and D, total MAP kinase staining. A and B, area of tumor with no activated MAP kinase in neoplastic cells. C–F, area of tumor with elevated activated MAP kinase. E, anti-phospho-MAP kinase antibody preincubated with dually phosphorylated immunizing peptide; F, anti-phospho-MAP kinase antibody preincubated with nonphosphorylated peptide. Original magnification, ×400.
tion in cell proliferation (18). The data reported here show elevated levels of active MAP kinase in high-grade and advanced stage prostate tumors. These observations, combined with previous reports demonstrating increased expression of cell proliferation markers as prostate cancer progresses to a more advanced and androgen-independent disease, suggest that activation of MAP kinase in prostate cancer is linked to cell proliferation (5).

Although the role of MAP kinase in cell proliferation has been established in the literature, our data suggest that elevated MAP kinase activation may also be important in the acquisition of androgen-independent prostate cancer growth. Analysis of two patients’ tumor samples showed no activation of MAP kinase before androgen ablation therapy and high levels of activated MAP kinase following androgen ablation treatment, suggesting that MAP kinase is activated in hormone refractory tumors.

Other studies have suggested that growth factor receptor signaling may play a role in the development of androgen-independent prostate cancer, supporting a possible role for MAP kinase activation in the development of androgen refractory prostate tumors. Voeller et al. (19) showed that activation of Ras is sufficient to induce androgen-independent growth of prostate cancer cells; expression of an activated v-Ras in the androgen-responsive LNCaP prostate cancer cell line enabled growth in the absence of androgen (19). This suggests that activation of Ras signaling can facilitate the progression of prostate cancer from an androgen-dependent to an androgen-independent state. Because Ras mutations are uncommon in prostate tumors, we propose that the transition from paracrine to autocrine loops of growth factor production and signaling during prostate cancer progression results in the chronic stimulation of the Ras pathway which helps drive prostate cancer cells to an androgen-independent state. Our observation of elevated levels of activated MAP kinase in high-grade and advanced-stage prostate tumors is consistent with chronic Ras signaling.

Previous studies examining the influence of growth factor signaling on AR activity have provided clues as to how continual stimulation of signal transduction pathways may affect androgen-independent growth. Culig et al. (20) demonstrated that IGF-I, EGF, and keratinocyte growth factor were able to induce AR-mediated reporter gene transcription using DU145 cells, a prostate cancer cell line that expresses neither AR nor PSA, and a cotransfection assay with an AR expression vector and chloramphenicol acetyltransferase reporter constructs (20). Growth factor-induced reporter gene expression was dependent on cotransfection of the AR expression construct and was blocked by the AR antagonist casodex. This suggests that activation of Ras signaling may play a role in the development of androgen-refractory prostate tumors. In the same study, activation of endogenous AR by IGF-I in LNCaP cells was demonstrated using endogenous PSA production as a marker. Again, the effect of IGF-I on PSA production was blocked by casodex. These experiments suggest that growth factor signaling can regulate androgen responsive genes by a mechanism that is AR dependent and androgen independent. This parallels discoveries on estrogen receptor regulation in genes by a mechanism that is AR dependent and androgen independent state. Progression results in the chronic stimulation of the Ras pathway which helps drive prostate cancer cells to an androgen-independent state. Our observation of elevated levels of activated MAP kinase in high-grade and advanced-stage prostate tumors is consistent with chronic Ras signaling.

### Table 1

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>Phospho-MAP kinase stainingb</th>
<th>n</th>
<th>Low (&lt;10%)</th>
<th>High (≥10%)</th>
</tr>
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<tr>
<td>2-8</td>
<td>18</td>
<td>6</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>2</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>8–10</td>
<td>23</td>
<td>2</td>
<td>5</td>
<td>16</td>
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a P < 0.001 by rank test for trend of increasing activated MAP kinase with increasing Gleason score.
b Scored as a percentage of tumor cell nuclei staining positive for phospho-MAP kinase.

Additional studies by Nazareth and Weigle (22) demonstrated that the AR can be activated by a protein kinase A activator in the absence of androgen (22). This activation can be blocked by a protein kinase A inhibitor peptide and the AR antagonists casodex and flutamide, indicating that the activation effect was due to PKA and dependent on AR. More recently it has been demonstrated that neuropeptides, which activate PKA, sensitize prostate cancer cells to signaling by peptide growth factors, such as EGF (6).

Thus, we propose that during prostate cancer progression and androgen ablation therapy, there may be a microenvironment in which peptide growth factors and neuropeptides activate signal transduction pathways that influence AR activity and help drive prostate cancer cells to an androgen-independent state. Our observation of elevated levels of activated MAP kinase in advanced prostate tumors begins to address the mechanism of androgen-independent growth of prostate tumors cells and suggests potential targets for blocking or reversing progression to androgen independence.

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### References

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