ABSTRACT

Skeletal metastases are common in advanced prostate cancer, causing considerable morbidity, and they are usually osteoblastic in nature with no clear explanation for this phenomenon. Bone morphogenetic proteins (BMPs) induce bone formation in vivo, and preliminary work showed a possible association between BMPs and prostatic skeletal metastases; differential expression favors BMP-6 as a potential new marker and mediator of osteoclastic deposit formation. We investigated BMP-6 mRNA and protein expression by in situ hybridization and immunohistochemistry in malignant and benign prostates from 40 men. BMP-6 mRNA expression was detected exclusively in malignant epithelial cells in 20 of 21 patients (95%) with metastases and in 2 of 11 patients (18%) with localized cancer, and it was absent in 8 benign samples. Immunostaining for BMP-6 was predominantly cytoplasmic and was present in all primary tumors with established metastases and in 4 of 11 (36%) organ-confined cancers. In benign prostatic hyperplasia, basal cells and areas of basal cell hyperplasia were positive for BMP-6 by immunohistochemistry. The results suggest a close association between BMP-6 expression in primary malignant prostatic tissue and skeletal metastases. BMP-6 may be responsible, in part, for the osteoblastic changes in metastatic lesions secondary to prostate cancer.

INTRODUCTION

The term BMP refers to an activity derived from bone that induces ectopic bone formation in vivo (1). The proteins belong to the TGF-β superfamily, and to date, 15 different BMPs have been identified (2). Since their discovery in 1965, researchers have focused their attention on identification and purification of these proteins and, more recently, on understanding the role of BMPs in normal human embryonic development. The proteins have a variety of functions in embryogenesis, including normal limb, skin, kidney, and heart development (3, 4). Their osteoinductive ability in vivo has stimulated the development of potential therapeutic applications in reconstructive orthopedic, periodontal, and craniofacial surgery.

To date, very few efforts have been made to link BMP activity with the development and progression of cancer. This is not surprising because the majority of bony secondaries result in osteolytic lesions, with increased bone resorption and osteoclastic activity. However, prostatic adenocarcinoma commonly metastasizes to the axial skeleton and exhibits a predominance of osteosclerotic lesions with increased osteoblastic activity. Prostate cancer is the second most common male malignancy in Europe, with over 85,000 cases registered every year (5). In the United States, it is the most common malignancy in males, with an estimated 317,000 new cases diagnosed in 1996 (6). Skeletal metastases represent the most common cause of morbidity in men with advanced prostate cancer, and there is no clear explanation for their osteoblastic nature. Several studies have demonstrated that benign and malignant prostate cells express a number of growth factors, including TGF-β, plasminogen activator, fibroblast growth factor, and epidermal growth factor (7, 8). None of these factors has been specifically linked to osteoclastic metastases. Pilot work, using RT-PCR to detect mRNA for BMPs 1–6, showed differential expression in benign and malignant prostatic tissue; BMP-6 was expressed in over 50% of primary tumors with established bony secondaries (9). Three other studies investigated BMP expression in human and rat prostate and in prostate cancer cell lines by Northern analysis, RT-PCR and immunohistochemistry, demonstrating different patterns of gene and protein expression, with slight elevation of BMP-6 mRNA in human prostate cancer compared with benign prostatic tissue (10–12). To date, however, the cells expressing the genes in the human prostate have not been identified. The aim of the present study was to investigate BMP-6 specifically, using the following methods: (a) ISH, to detect mRNA for BMP-6 and to identify the phenotype of cells expressing the gene in prostates of men with metastatic disease, in prostates of men with organ-confined cancers, and in men with BPH; and (b) immunohistochemistry to correlate gene and protein expression, using a novel mouse antihuman monoclonal antibody for BMP-6.

PATIENTS AND METHODS

Patients. Forty patients were investigated. Thirty-two men had newly diagnosed and untreated prostate cancer, and 8 had BPH. All cancer patients were clinically staged using digital rectal examination, serum PSA estimation (HybriTech assay), transrectal ultrasonography, and isotope bone scanning. Twenty-one had evidence of skeletal metastases, and 11 had apparently localized cancer. Eight of the 11 patients with apparently organ-confined disease underwent radical prostatectomy and bilateral pelvic lymphadenectomy. The remaining three patients were observed.

Tissue Sample Preparation. Archival tissue was obtained from transurethral resection specimens, needle core biopsies of the prostate, or radical prostatectomy specimens. The tissue was fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections were cut at 3 μm, mounted onto 3-aminopropyltriethoxysilane-coated slides, and dried overnight at 37°C followed by 1 h at 56°C. BMP-6 Riboprobes. A 757-bp fragment of the human BMP-6 cDNA, representing bases 1019–1776 of the published sequence, was amplified by PCR using plasmid cDNA as the template with the following oligonucleotides: 5'-CTCTGACCTGTTTGTGGTG-3' and 5'-CTTCCGGTTTTTTTAAAGGC-3'. The fragment was cloned in forward and reverse orientation into the pBluescript SK+ vector (Stratagene). DNA sequencing analysis was performed on this fragment and showed it to be identical to the published BMP-6 cDNA sequence (GenBank accession no. M38694; Ref. 13). Recombinant plasmids were linearized with Smal and gel purified from low melting point agarose using standard conditions. The purified DNA was subsequently phenol extracted under RNase-free conditions, ethanol precipitated, and resuspended in diethylpyrocarbonate-treated water. Sense and antisense riboprobes were synthesized by in vitro transcription using T7 RNA polymerase and the
RESULTS

ISH. mRNA for BMP-6 was detected exclusively in the cytoplasm of malignant prostatic epithelial cells, with no signals in the prostatic stroma (Fig. 1a). None of the BPH samples or benign prostatic tissue present in sections containing prostate cancer showed any evidence of BMP-6 mRNA expression.

Twenty of 21 patients (95%) with proven skeletal metastases showed cytoplasmic signals for BMP-6 mRNA, compared with 2 of 11 patients (18%) with apparently localized cancer (P < 0.0001). The first of these patients was 47 years old, had a strong family history of prostate cancer, and received radical prostatectomy. He had T<sub>2</sub>N<sub>0</sub>M<sub>0</sub> disease, a Gleason score of 7, negative surgical margins, and a preoperative serum PSA level of 27 ng/ml. His serum PSA remained undetectable 18 months after surgery. The second patient was 60 years old and also underwent radical prostatectomy. His preoperative PSA was 5.6 ng/ml; he had T<sub>3</sub>N<sub>0</sub>M<sub>0</sub> disease, a Gleason score of 7, and positive surgical margins; and his serum PSA was slowly rising (1.4 ng/ml) 24 months after surgery. His bone scan remained negative.

Western Blotting. The specificity of the antibody used for immunohistochemistry was confirmed by Western analysis performed on two established prostate cancer cell lines, PC-3 and DU 145 (Fig. 2). This demonstrated a single band with a molecular weight of approximately 64,000 in both cell lines. There were no bands in the benign prostatic stroma representing negative controls (data not shown).

Immunohistochemistry. All primary tumors with established metastatic disease showed positive cytoplasmic staining for BMP-6 in malignant prostatic epithelial cells, compared to 4 of 11 apparently localized cancers (P < 0.0001). These included the two patients with mRNA for BMP-6 as shown by ISH, and two other patients who underwent radical prostatectomy with negative margins, organ-confined disease, and an undetectable serum PSA level 2 and 3 years postoperatively. When present, immunostaining was diffuse throughout the tumors (Fig. 1b), and was scored by two observers (M. C. R. and P. A.) according to its intensity, ranging from 1+ to 4+. There was no correlation between intensity of staining, tumor stage, and serum PSA levels (data not shown). However, in high-grade tumors with Gleason scores of 8–10, intensity of staining was low (1+; Fig. 1c). In addition, five tumors exhibited faint nuclear staining. Areas of high-grade prostatic intraepithelial neoplasia were present in eight cancers, and seven of these areas were positive for BMP-6. In samples from BPH tissue, basal cells and areas of basal cell hyperplasia were positive for BMP-6 (Fig. 1d).

Further clinical details in relation to BMP-6 expression are listed in Table 1.

DISCUSSION

In this study, we used ISH and immunohistochemistry to examine the distribution of BMP-6 mRNA and protein in samples of human benign prostatic tissue and prostatic adenocarcinoma. The specificity of the riboprobes used for ISH was confirmed using Northern analysis, demonstrating a single transcript of approximately 4 kb as described previously (Ref. 10; data not shown). This was significantly different from the closely related BMP-5 and BMP-7, which show approximately 70% homology to BMP-6 but have transcript sizes of 2.8 and 2.4 kb, respectively (13, 18). The specificity of the novel BMP-6 monoclonal antibody used in the study was confirmed by Western blotting.

The findings confirm that BMP-6 mRNA is exclusively expressed in malignant prostatic epithelial cells and is predominantly present in primary tumors with established skeletal metastases. BMP-6 protein was also localized in the cytoplasm of malignant prostatic epithelial
IMMUNOLOCALIZATION AND mRNA EXPRESSION OF BMP-6

Fig. 1. a, photomicrograph of ISH studies on a section of prostatic adenocarcinoma, showing strong cytoplasmic signals in malignant epithelial cells with antisense BMP-6 probe. ×400. b, photomicrograph of immunohistochemical studies on a section of prostatic adenocarcinoma, showing strong cytoplasmic staining for BMP-6 in malignant epithelial cells and weaker staining in basal cells of adjacent benign glands. ×200. c, photomicrograph of immunohistochemical studies of a section from high-grade prostatic adenocarcinoma, illustrating the pattern of weak cytoplasmic staining for BMP-6 found in epithelial cells of these tumors. ×400. d, photomicrograph of immunohistochemical studies on a section of BPH, showing cytoplasmic staining for BMP-6 in basal cell layers and areas of basal cell hyperplasia. ×200.

cells, with occasional nuclear staining, showing a good correlation with ISH findings. In addition, the protein was detected in basal cell layers and areas of basal cell hyperplasia in both benign and malignant tissue. A study by Harris et al. (10) reported variable degrees of expression of mRNA for BMP-2, -3, -4, and -6 by Northern analysis in prostate cancer cell lines, rat ventral prostate, and RNA extracts from four normal human prostates. Their findings suggested that BMP-4 was predominantly expressed in the normal human prostate, but no human prostate cancer or BPH samples were analyzed. More recently, the same group reported BMP-3 mRNA expression in fetal rat calvarial osteoblasts, rat prostate adenocarcinoma cells, and the human prostate cancer line PC-3. Analysis of three different clones of BMP-3 showed that the nucleotide sequence of BMP-3 was identical in human bone cells and prostate cancer cells, with degrees of variation in the pro- or precursor region between the two species (12). A further study by Barnes et al. (11) analyzed BMP-6 expression in rat and human cell lines, human benign tissue, and human malignant tissue by immunohistochemistry and RT-PCR. Their results showed BMP-6 expression in all of the samples analyzed and a higher level of BMP-6 mRNA and protein in human prostate cancer compared with normal tissue. However, the number of patients studied is not stated, and RT-PCR was performed in the absence of controls, which does

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not allow accurate quantification of BMP-6 mRNA expression. Although our ISH findings are in accordance with their immunohistochemical results regarding malignant cells, we have not been able to detect mRNA for BMP-6 in BPH, and immunostaining with our BMP-6 antibody showed staining only in basal cell layers of benign prostatic tissue. This may reflect the fact that in their study, benign tissue was obtained from areas adjacent to carcinoma, rather than from patients with BPH only, as was done in our study. Alternatively, in our study, basal cell immunostaining in the absence of BMP-6 mRNA signals could be accounted for by differences in the respective half-lives of the protein and mRNA species. In addition, basal cells may have low BMP-6 mRNA levels detectable by the sensitive RT-PCR method, but not by ISH, with high protein levels leading to positive immunostaining. Our immunohistochemical findings highlight the importance of basal cells in the human prostate. This population of cells has been advocated as having specific properties in the progression of benign prostatic cells to the malignant phenotype. In a study investigating the differential proliferative function of basal compared with secretory cells in the prostate, Bonkhoff et al. (19, 20) demonstrated that the majority of proliferating cells in atypical hyperplasia that progressed to invasive carcinomas were localized in the basal cell layers. This may indicate that the basal cell layer represents the main proliferative compartment of the prostatic epithelium in normal, benign hyperplastic and neoplastic conditions, and would explain the fact that basal cells express neoproteins that may contribute to the biological behavior of developing tumors.

Vgr-1 (the murine equivalent of BMP-6) expression has been observed in nonskeletal tissue in embryonic, newborn, and adult mice, and it has been suggested that the coordinated expression of this and other members of the TGF-β superfamily is required to control the progression of specific cell types through their differentiation pathways (3). Human BMP-6 has also been identified in placenta and brain cDNA libraries (13). In a study investigating the effects of BMP-2, BMP-4, and BMP-6 on differentiation of rat osteoblasts in vitro, it was shown that although the three BMPs could stimulate bone-nodule formation, nodule size and number of cells per nodule were increased with BMP-6 only (21). These findings support the hypothesis that BMP-6 may have a relevant role in the osteoblastic reaction found in skeletal metastases secondary to prostatic adenocarcinoma. In our study, mRNA for BMP-6 was absent in patients with BPH and present in only 2 of 11 men with nonmetastatic prostate cancer. These patients were interesting in that the first had features of hereditary prostate cancer, with early onset, two first-degree relatives who had died of metastases, and a serum PSA of 27 ng/ml, suggesting disseminated disease, despite a negative bone-scan. Although his serum PSA was undetectable 12 months postoperatively, he is being carefully followed up. The second patient had clinically organ-confined disease at diagnosis, but was found to have locally advanced prostate cancer pathologically, and showed signs of treatment failure with biochemically recurrent disease 18 months after surgery. The only patient with metastatic disease and no BMP-6 expression in his primary tumor appeared to have had low-volume disease, with no evidence of local extension, and a modest rise in his serum PSA (18 ng/ml) compared with the majority of patients with skeletal secondaries (median, 100 ng/ml). The two patients with localized disease who had positive staining for BMP-6 by immunohistochemistry but no mRNA expression have not progressed to date, although follow-up is relatively short. The results of our study, showing that BMP-6 expression was found in the majority of patients with skeletal metastases, are in accordance with the previous work by Bentley et al. (9), who showed by RT-PCR that BMP-6 was selectively expressed in the primary tumor of bone-scan positive patients and absent in nonmetastatic tumors, benign tissue, and ocular melanomas, which rarely metastasize to bone. In addition, our study appears to be the first to localize BMP-6 mRNA in malignant prostatic epithelial cells. It would be of importance to investigate the presence of BMP-6 in metastatic specimens, but we have not been able to obtain matching tissue samples for these studies. Despite the close association between BMP-6 expression and skeletal metastases, the data generated by our study do not allow us to determine whether BMP-6 has any role in the initiation of skeletal secondaries or whether it is in part responsible for their osteoblastic nature. This warrants further investigation of BMPs (and BMP-6 in particular), which may have a key role in the mechanisms of skeletal metastases in prostate cancer.

**REFERENCES**


Immunolocalization and Messenger RNA Expression of Bone Morphogenetic Protein-6 in Human Benign and Malignant Prostatic Tissue

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