Reduced Lysyl Oxidase Messenger RNA Levels in Experimental and Human Prostate Cancer

Chengzhen Ren, Guang Yang, Terry L. Timme, Thomas M. Wheeler, and Timothy C. Thompson

Scott Department of Urology [C. R., G. Y., T. L. T., T. M. W., T. C. T.] and Departments of Pathology [T. M. W.], Cell Biology [T. C. T.], and Radiology [T. C. T.], Baylor College of Medicine, Houston, Texas 77030

ABSTRACT

To identify genes associated with prostate cancer progression, we developed a strategy involving the use of differential display PCR and a panel of genetically matched primary tumor- and metastasis-derived mouse prostate cancer cell lines. We analyzed sequences that were differentially stimulated by transforming growth factor-β1 in primary tumor-versus metastasis-derived cell lines, based on our previous studies indicating that acquisition of differential responses to this growth factor could result in phenotypic traits that facilitate tumor metastasis from specific cell clones within the primary tumor. Using this system, we isolated and sequenced a cDNA fragment that encoded mouse lysyl oxidase (LO) and was induced by transforming growth factor-β1 in primary tumor but not in metastasis-derived cells. Northern blotting analysis revealed increased LO expression in a panel of primary tumor cell lines but significantly reduced or nondetectable expression in their matched metastatic counterparts. Further in situ hybridization analysis revealed LO expression in normal mouse prostate epithelium but, in most cases, progressive loss of expression in primary prostate cancer and associated metastatic lesions. Importantly, in situ hybridization studies of normal human prostate and prostate malignancies revealed a similar loss of expression during progression to metastasis. The progressive loss of LO expression during prostate cancer progression provides insight into the mechanisms that underlie this disease. In addition, LO may provide a useful molecular marker and/or establish a novel therapeutic target for prostate cancer.

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in men in the United States (1), and the ability to treat this malignancy relates to the degree of progression, with those cancers that are metastatic having a much poorer prognosis. To better understand the molecular pathways associated with metastasis, we developed a model system involving panels of genetically matched cell lines derived from primary and metastatic mouse prostate tumors. The malignant tissues were produced using the MPR1 model system and involved the induction of metastatic prostate cancer in vivo by the transduction of the ras and myc oncogenes into fetal prostate tissues from p53 knockout mice (2). Multiple sets of early-passage clonal cell lines from both primary and metastatic tumor foci recovered from the same inbred experimental animal were established. Because these experiments are performed in inbred mouse strains and conditions for outgrowth of both primary and metastasis-derived cell lines are closely controlled, these cell line systems are both genetically and biologically matched, such that the predominant genetic differences between these cell lines should be related to the metastatic process. We previously identified TGF-β1 as being associated with prostate cancer (3, 4). We further demonstrated that tumor cell lines derived from lung metastases secreted relatively larger amounts of total TGF-βs and lost most or all TGF-β1 growth inhibition but responded to TGF-β1 through induction of the type IV collagenase matrix metalloproteinase-9, whereas cell lines derived from primary tumors secreted relatively smaller amounts of total TGF-βs and retained TGF-β1 growth inhibition but lacked TGF-β1-induced collagenase activity (5). Synthesis of another extracellular matrix protein, PAI-1, was stimulated by TGF-β1 in both primary and metastatic cell lines. Because TGF-β1 regulates a wide variety of genes that may be involved in the metastatic process, we adapted the DD-PCR technique (6–8) to detect the downstream gene targets for TGF-β1 that may underlie the selection of metastatic clones. Initially, genes that are regulated by TGF-β1 in primary tumor-derived prostate cancer cells are detected by DD-PCR. Cloned cDNA fragments are used to screen primary versus metastatic mouse prostate cancer cell lines for sequences that are differentially regulated by TGF-β1 by Northern blot analysis. Sequences that are differentially regulated by TGF-β1 are then used to analyze constitutive gene expression by Northern blot analysis using a large panel of primary and metastatic mouse prostate cancer cell lines.

One gene that was determined to be differentially regulated by TGF-β1 and also demonstrated reduced constitutive expression in metastatic cells relative to primary cells was the LO gene. LO is a copper-dependent enzyme that is expressed predominantly in bone, blood vessels, and connective tissue and cross-links collagen and elastin in the extracellular matrix, leading to stabilization of matrix structure (9, 10). Expression of LO has been reported to be stimulated by TGF-β1 in osteoblasts, vascular smooth muscle cells, and lung fibroblasts (11–13). Increased LO activity has been reported in fibrotic disorders, whereas decreased activity is associated with inherited disorders of collagen (14–16). Increased expression of the LO gene has also been reported in mouse fibroblasts, which revert from a ras-transformed phenotype to a normal phenotype (17, 18), and it was recently demonstrated that LO is regulated by the antioncogenic transcription factor IRF-1 (19), suggesting that LO may behave as a tumor suppressor. Low levels of LO mRNA expression by RT-PCR have also been reported in selected human tumor cell lines (20, 21), and recently, LO expression has been detected in the extracellular matrix of breast cancer (22).

Here, we document significant expression of LO in normal prostatic epithelium using in situ hybridization. LO mRNA was also detected in normal prostatic stroma, albeit at significantly lower levels. We further demonstrate that LO expression is progressively reduced in primary and metastatic prostate cancer. Because of its well-documented biological activities, significantly reduced LO expression may play a role in prostate cancer progression.

MATERIALS AND METHODS

Cell Lines. Mouse prostate cancer cell lines were derived from primary tumors or metastatic deposits in the same host animal implanted with a ras+myc-initiated p53-nullizygous MPR (2). The cell lines were analyzed for...
Fig. 1. A, schematic diagram of an approach to identifying TGF-β1-regulated genes. B, detection of LO induction by DD-PCR screening for TGF-β1-regulated genes in a mouse prostate cancer cell line. Portions of the DD-PCR gel comparing mRNA from 148-1PA cells treated with (Lanes +) or without (Lanes −) TGF-β1 in SFM for 12 h, primed with three different primers: P10, P11, or P12. Arrow, TGF-β1-induced band, which was cloned and sequenced to reveal homology to a portion of the LO gene. C. Northern blot screen of total RNA from the primary tumor-derived cell line 148-1PA and a cell line derived from a lung metastasis 148-1LMD that was clonally identical for retroviral integration by Southern blot analysis (2). Cells were treated for either 12 or 24 h with TGF-β1 in SFM. The LO probe detected up-regulation by TGF-β1 at both 12 and 24 h in 148-1PA cells. In 148-1LMD cells, the constitutive level of LO was barely detectable, and no significant increase was observed in TGF-β1-treated cells. The blot was stripped and reprobed with PAI-1, demonstrating up-regulation by TGF-β1 at both 12 and 24 h for both 148-1PA and 148-1LMD cells. Equivalent RNA loading was verified by stripping the blot and reprobing it with a rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe.
RESULTS

Detection of LO by DD-PCR and Demonstration of Differential Response to TGF-β1 Stimulation in Primary Tumor- and Metastasis-derived Mouse Prostate Cancer Cells. To isolate genes that are differentially induced or repressed in primary prostate cancer cells compared to metastatic cells following TGF-β1 stimulation, we initially treated a primary mouse prostate cancer cell line, 148-1PA (5), with 2 ng/ml TGF-β1 in vitro and proceeded with a modified DD-PCR protocol (Refs. 6–8; Fig. 1A). In this modification, we substituted the oligo(dT) primer with a semiarbitrary 10-mer for the initial reverse transcription and used this single 10-mer for subsequent amplification steps. Each primer amplifies a large number of primer-specific bands that are similarly expressed in either the presence or absence of TGF-β1, but in some cases, bands are induced or repressed in the 148-1PA cells by TGF-β1 stimulation. Several of the differentially regulated DD-PCR fragments were cloned, sequenced, and compared to the known databases for both mouse and human genes. In approximately 90% of the cases, it was apparent that we had cloned a portion of a known mouse gene or a mouse homologue for a human gene. One of the fragments detected as induced in 148-1PA cells is shown in Fig. 1B. Using primer 11, increased levels of this fragment were detected in TGF-β1-treated 148-1PA cells, and the fragment was further isolated, cloned, and sequenced. Upon comparison with the available databases (GenBank Version 86.0), the 308–307-bp fragment was 100% identical (excluding three mismatched primer sequences) with the mouse LO gene and 93% identical with the human LO gene. The cloned fragment was then used in experiments with both the primary mouse prostate cancer cell line 148-1PA and its matched metastatic counterpart, 148-1LMD, to analyze and compare the extent of induction of LO by TGF-β1. LO was induced significantly at both 12 and 24 h poststimulation in 148-1PA cells but not in 148-1LMD cells, in which no induction was seen and constitutive levels were nearly undetectable (Fig. 1C).
In contrast to LO, induction of PAI-1 (23) mRNA levels by TGF-β1 was observed at both 12 and 24 h poststimulation in both 148-1PA and 148-1LMD cells. Further analysis of a panel of primary and metastatic cells derived from three independent animals (2, 5) revealed that LO expression was readily detected in five of six primary mouse prostate cancer cell lines but only two of seven metastatic cell lines (Fig. 2). Endo B (cytokeratin 18; Ref. 24) mRNA, a marker for normal prostatic epithelium and prostate cancer cells (25), was present at variable levels in all mouse prostate cancer cell lines, documenting the epithelial origin of the cell lines. Overall, these results suggested that LO represented a gene that may be stimulated by TGF-β1 in nonmetastatic prostate cancer; yet, during progression to metastasis, clones were selected for loss of TGF-β1 stimulation, and reduced LO expression was an acquired feature of the metastatic phenotype.

Analysis of LO mRNA in Normal and Malignant Mouse Prostate Tissues by in Situ Hybridization. To analyze mRNA levels in normal and malignant mouse prostate, the 308-307-bp LO fragment that was originally cloned by DD-PCR from 148-1PA cells was used for in situ hybridization analysis of normal, primary tumor, and metastatic mouse prostate cancer tissues (Fig. 3). The results indicated that significant mRNA levels were present in normal mouse prostate epithelium (Fig. 3A). LO mRNA was also detected in the stroma of normal mouse prostate but at a significantly reduced level. In a matched set of primary (Fig. 3C) and metastatic (Fig. 3D) mouse prostate cancer tissues, in situ hybridization analysis revealed significantly reduced levels of LO mRNA in primary mouse prostate cancer relative to normal mouse prostate epithelium and, further reduced levels of LO expression in metastatic lesions.

Analysis of LO mRNA in Normal and Malignant Human Prostate Tissues by in Situ Hybridization. To investigate LO mRNA levels in normal and malignant human prostate, we analyzed tissue sections using in situ hybridization with the 308-307-bp mouse fragment of mouse LO DD-PCR fragment (Fig. 4). This fragment is 93% homologous with the human LO gene and was, therefore, suitable for such studies. The results of the analysis of multiple normal and malignant tissues revealed a pattern similar to that seen in the mouse. Significant levels of LO mRNA were detected in benign prostate epithelium (Fig. 4A). LO was also detected in normal prostatic stroma, but at a significantly reduced level. In primary human prostate cancer, LO mRNA levels were reduced relative to that seen in benign prostatic epithelium (Fig. 4, A and C; Table 1), and analysis of metastatic specimens revealed a further diminution of LO mRNA (Fig. 4D; Table 1).

DISCUSSION

Here, we describe a novel approach for identifying genes that are differentially regulated by TGF-β1 during prostate cancer progression. We have previously established sets of early-passage cell lines from the primary tumor and associated metastases from multiple animals that were induced in vivo by the transduction of the ras and myc oncogenes into fetal prostate tissues from p53 knockout mice (2). Further studies demonstrated that metastasis-derived cell lines se-
created relatively greater quantities of total TGF-βs and lost most or all TGF-β1 growth inhibition but responded to TGF-β1 through induction of the type IV collagenase matrix metalloproteinase-9, whereas primary tumor-derived cell lines secreted relatively lesser quantities of total TGF-βs and retained TGF-β1-induced growth inhibition but lacked TGF-β1-regulated collagenase activity. These studies and others led us to believe that abnormal biological responses to TGF-β1 played an important role in prostate cancer progression. To pursue the identification of genes that may lose normal response to TGF-β1 during prostate cancer progression, we developed a novel approach using DD-PCR in combination with stimulation of gene expression in primary tumor- versus metastasis-derived cell lines by TGF-β1 (Fig. 1A). The first gene identified that was induced by TGF-β1 in a primary tumor-derived cell line but not in its genetically matched metastatic counterpart was LO. Interestingly, other TGF-β1-induced genes, such as PAI-1, were stimulated to a similar extent in both cell lines, as reported previously (5).

To our knowledge, this is the first report documenting LO expression in normal prostate or prostate cancer. Our studies clearly revealed the presence of LO mRNA predominantly in epithelium and, to a lesser extent, in the stromal cells of normal mouse and human prostate tissues. This new information adds to a limited understanding of the localization of LO and glandular tissues in general. Previous studies on LO expression and regulation have been done using mainly stromal-derived cell lines (11-13, 17-21). More recently, studies of LO expression were reported for normal and malignant human breast tissues (22). Here, LO was undetectable in normal breast tissue using immunostaining for LO protein. However, in situ hybridization analysis for LO demonstrated that the presence of mRNA in stromal cells surrounding glandular epithelium and reduced LO expression was associated with loose or scirrhous stroma that accompanied invading tumors (22). Our studies also associated loss of LO expression with prostate cancer progression. In the case of prostate cancer, the malignant cells per se demonstrate a progressive reduction in LO mRNA during primary tumor development and metastasis. Studies are currently ongoing to further define the compartmentalization of LO in normal and malignant prostate and to better understand its biological and clinical significance. This result further validates our differential cloning strategies and provides a novel progression/metastasis-related candidate gene for further studies. Although our results indicate loss of LO mRNA during mouse and human prostate cancer progression, the molecular basis for this loss is not clear at this time. It is possible that alterations occur not only in the LO gene but also in the antioncogenic transcription factor, IRF-1, which regulates its expression (19).

| Relative LO mRNA levels in human prostate tissues were scored as negative (−) to strongly positive (+++), depending on the fluorescence intensity on tissue sections. |
| n, number of specimens examined. |

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Our results have implications for the biological underpinnings of prostate cancer progression. It has been previously suggested that the protective effect of a collagenized matrix might be determined by collagen cross-linking that could dictate fibrillar and basement membrane stability against metalloproteinase activities (26). This concept was recently supported by studies involving invasive ductal breast carcinoma (22), and now, it is supported by this study in prostate cancer. This protective effect may underlie, in part, the phenomenon associated with the reversion of ras-transformed 3T3 cells by LO (17, 18). In addition to promoting a physical barrier for malignant progression through extracellular matrix cross-linking, it is also conceivable that maintenance of the integrity of the extracellular matrix prevents the release of mitogenic growth factors (27). These concepts should now be considered relevant for prostate cancer progression. Progressive loss and/or unusual patterns of LO expression in prostate cancer biopsy material may be of prognostic significance. The LO gene should now be considered a potential tumor suppressor gene or component of a tumor suppressor pathway for prostate cancer and, therefore, a target for molecular therapeutic strategies.

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