Secreted Caveolin-1 Stimulates Cell Survival/Clonal Growth and Contributes to Metastasis in Androgen-insensitive Prostate Cancer

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Introduction

Caveolin-1 is an integral protein of caveolae, known to play important roles in signal transduction and lipid transport. We demonstrate that caveolin-1 expression is significantly increased in primary and metastatic human prostate cancer after androgen ablation therapy. We also show that caveolin-1 is secreted by androgen-insensitive prostate cancer cells, and that this secretion is regulated by steroid hormones. Significantly, caveolin-1 was detected in the MDL fraction of serum specimens from patients with advanced prostate cancer and to a lesser extent in normal subjects. Conditioned media from high passage caveolin-1 secreting, androgen-insensitive, LNCaP cells stimulated increased viability and clonal growth of low passage, caveolin-1-negative, androgen-sensitive, LNCaP cells in vitro, and this effect was blocked by treating the media with caveolin-1 antibody. i.p. injections of caveolin-1 antibody suppressed the orthotopic growth and spontaneous metastasis of highly metastatic, androgen-insensitive caveolin-1-secreting mouse prostate cancer. Overall, our results establish caveolin-1 as an autocrine/paracrine factor that is associated with androgen-insensitive prostate cancer. We demonstrate the potential for caveolin-1 as a therapeutic target for this important malignancy.

Materials and Methods

Patients and Specimens. 61 Stage D prostate cancer patients were included in this study. For each patient, one primary prostate cancer and one or more metastatic cancer specimens from different organs were obtained either at the time of radical prostatectomy or at autopsy. From the 11 hormone-refractory patients, a total 33 metastases were derived from lymph node (n = 12), lung (n = 8), bone (n = 1), liver (n = 5), adrenal gland (n = 1), bladder (n = 2), brain (n = 1), and soft tissue (n = 3). Fifty-five metastases were obtained from the 50 nontreated patients, which included lymph node (n = 48), lung (n = 2), bone (n = 2), liver (n = 1), bladder (n = 1), and soft tissue (n = 1). Tissues were fixed in 10% formalin and embedded in paraffin according to a routine procedure. Six-μm sections were made from the tissue blocks, and some were stained with H&E for morphological evaluation. They were immunostained using a polyclonal cav-1 antibody and the avidin-biotin complex procedure previously described (4). The immunostained sections were evaluated at a power of ×200 under a Zeiss microscope. For each specimen, the whole cancer area was scanned. Positive cav-1 staining was defined as the presence of any microscopic field in which cancer cells gave rise to cav-1-positive granular immunoreaction products in their cytoplasm. Serum samples were obtained from five patients with radiorecurrent prostate cancer and four healthy individuals. Serum lipoproteins were separated into lipoprotein sub-fractions by KBr density gradient ultracentrifugation following a modified method of Redgrave et al. (9).

Cell Culture. Mouse prostate cancer cell lines derived from primary tumors (148-1PA and 151-1PA) or metastatic deposits (178-2BMA, 148-1LMD, 151-2LMB, 151-2LMC, and 151-1LM1), were cultured as described previously (4). The human cav-1 cDNA in pcDNA3.1 was transfected into LNCaP cells with Tfx reagent (Promega, Madison, Wisconsin; #151-2LMB, 151-2LMC, and 151-1LM1), were cultured as described previously (4). The growth media for the different cells was in 10% fetal bovine serum as follows: RPMI 1640 for LNCaP; MEM-NEAA for DU145; F12K for PC3; F12K supplemented with heparin and endothelial cell growth supplement for human umbilical vascular endothelial cell; and DMEM for human intestinal smooth muscle and all mouse cells. The human cav-1 cDNA in pcDNA3.1 was transfected into LNCaP cells with Tfx reagent (Promega, Madison, Wisconsin; Ref. 7). For conditioned medium preparation, subconfluent cultures were washed three times with PBS and incubated with SFM for 24 h; the media was collected and contamination of membranous cav-1 from cell debris was minimized by centrifugation at 1,000 × g and then at 100,000 × g. Conditioned media for in vitro viability assays (see below) was concentrated ×20 and treated with cav-1 antibody or IgG (10 μg/ml) and incubated for 4 h at 4°C.

Western Blot Analysis. Conditioned media was collected and centrifuged as described above and 1 ml concentrated by TCA precipitation. The precipitate was redissolved in 70 μl of SDS sample buffer and 30 μl were loaded per well. Proteins obtained from lysed cells and from TCA-precipitated conditioned media were separated by 12% SDS-PAGE, and transferred to nitrocel-
The frequency of cav-1-positive cancers in each group of hormone-sensitive prostate cancer patients was determined by scoring the percentage of positive cells in each microscopic field of tissue sections. The results showed that cav-1 was expressed in 62% of the metastatic tumors from hormone-naive patients (38%), but this increase was not significant (P > 0.05; Mann-Whitney test). Increased cav-1 positivity in hormone-refractory prostate cancer is consistent with previous reports that have demonstrated overexpression of cav-1 in multidrug-resistant prostate cancer. Cav-1 positivity in hormone-refractory prostate cancer is consistent with previous reports that have demonstrated overexpression of cav-1 in multidrug-resistant prostate cancer.

We used the mouse prostate cancer cell line 178-2BMA, which is derived from a mouse prostate metastasis generated from the metastatic mouse prostate reconstitution model (10) and HP-LNCaP cells to test the possible regulation of cav-1 secretion by DHT and Dex in vitro. Both cell lines were shown to be insensitive to androgen in vitro, i.e., no significant changes in cell number or viability were detected under serum-free conditions in the presence and absence of 10 nM testosterone (data not shown). The results showed that cav-1 was expressed and secreted into the medium. In contrast, nonprostatic cells such as endothelial, fibroblast, and smooth muscle had a substantial amount of intracellular cav-1 yet minimal or undetectable levels of cav-1 in their conditioned media (Fig. 1A). We used the mouse prostate cancer cell line 178-2BMA, which is derived from a mouse prostate metastasis generated from the metastatic mouse prostate reconstitution model (10) and HP-LNCaP cells to test the possible regulation of cav-1 secretion by DHT and Dex in vitro. Both cell lines were shown to be insensitive to androgen in vitro, i.e., no significant changes in cell number or viability were detected under serum-free conditions in the presence and absence of 10 nM testosterone (data not shown). The results showed that cav-1 was expressed and secreted into the medium. In contrast, nonprostatic cells such as endothelial, fibroblast, and smooth muscle had a substantial amount of intracellular cav-1 yet minimal or undetectable levels of cav-1 in their conditioned media (Fig. 1A).

We investigated the secretory route for cav-1 by expressing human cav-1 in cav-1-negative LP-LNCaP cells. After transfection, a substantial amount of ectopically expressed cav-1 was detected in the media compared with that in the cell lysate, and cav-1 secretion was increased in response to DHT. Cav-1 was not detected in the media or cell lysate of the vector control-transfected cells, yet all transfected cells expressed prostate-specific antigen into the media in a DHT-regulated fashion (Fig. 1C). These results show that cav-1 is secreted by androgen-insensitive mouse and human prostate cancer cells in response to specific steroid hormones. Although we do not provide evidence for the mechanism by which cav-1 enters the secretory pathway, the results show that ectopically expressed cav-1 is secreted by LNCaP cells, and that secreted cav-1 migrates on SDS-PAGE.
similarly to that derived from endothelial cells and fibroblasts, suggesting that the secreted form is not modified posttranscriptionally. To determine whether cav-1 could also be secreted by human prostate cancer cells in vivo, we fractionated human serum and analyzed various fractions for cav-1. Our results revealed that cav-1 is specifically detected in the serum HDL₂ lipoprotein subtraction, and that cav-1 levels may be higher in the serum of prostate cancer patients compared with the serum of normal individuals (Fig. 1D).

The function of secreted cav-1 was investigated by testing the effects of concentrated conditioned media collected from HP-LNCaP cells on LP-LNCaP cell viability and clonal growth under serum-free conditions. The results indicate that secreted cav-1 was capable of promoting viability, using a standard MTT method (Ref. 6; Fig. 2A) or luminescent technique (Packard ATPLite; Fig. 2B) and of stimulating viability/clonal growth using a clonogenic assay (Fig. 2C). To test whether such activities would be specific for the cav-1 molecule, polyclonal cav-1 antibody was added to conditioned media or rabbit IgG as a control. Treatment of the conditioned media with anti-cav-1 antibody reduced the viability significantly (P < 0.001 for MTT and clonogenic assays and P < 0.0001 for ATPLite assay) compared with the IgG-treated medium. We also tested the effect of secreted cav-1 on Tg-induced apoptosis in LP-LNCaP cells. Tg promotes apoptosis (14), characterized by caspase activation and the appearance of apoptotic bodies in these cells (data not shown). The results indicated that secreted cav-1 was able to protect the cells from the apoptotic effects of this drug (Fig. 2D). These studies revealed that media containing secreted cav-1 generates antiapoptotic activities in prostate cancer cells similar to those elicited after enforced expression of cav-1 within the cell (6, 7).

We then tested whether blocking secreted cav-1 activity in vivo with specific antibodies would result in therapeutic activity, potentially through abrogation of the antiapoptotic effects of secreted cav-1. Androgen-insensitive 178-2BMA cells that spontaneously metastasize with high frequency (nearly 100%) to lung, lymph nodes, and bone were grown as orthotopic tumors in adult male mice. After 21 days of treatment with cav-1 antibody or IgG, the animals were sacrificed. The mean tumor wet-weight (Fig. 3A) and the mean number of lung metastases (Fig. 3B) of the anti-cav-1-treated group was significantly lower than the IgG-treated group (P < 0.01 and P < 0.05, respectively). The cav-1 antibody-treated group also had a significantly lower percentage of cancer cell volume in lymph nodes (P < 0.01; Fig. 3C). The metastatic cell density in the bone marrow (Fig. 3D) was also reduced significantly (P < 0.05) in the cav-1 antibody-treated mice compared with those of the IgG-treated group. These results show that neutralization of secreted cav-1 in vivo by specific antibody suppresses primary prostate tumor growth and spontaneous metastasis to the lung, lymph nodes, and bone.

Overall, the results of this study contribute significantly to the understanding of androgen-insensitive prostate cancer. Previous studies have documented that bcl-2 overexpression may characterize a subset of androgen-insensitive disease (15). The aberrant expression of HER-2/neu has been implicated in androgen independence in animal models (16) and by immunohistochemical analyses of human specimens (17). However, as reviewed by Scher (18), the role of HER-2/neu in prostate cancer progression is not as self-evident as it is...
in breast cancer. In this study, we demonstrate that cav-1 up-regulation is associated with the development of androgen-insensitive prostate cancer, and that androgen-insensitive prostate cancer cells secrete biologically active cav-1 in a steroid-regulated fashion. We have shown that testosterone up-regulates cav-1 expression in prostate cancer cells, in part, through transcriptional activation. Therefore, in the presence of testosterone, cav-1 expression and/or secretion may be significantly stimulated in prostate cancer cells. Androgen ablation may select for alternative pathways of cav-1 regulation that could include glucocorticoid-stimulated cav-1 secretion, as shown here. It was shown previously that polypeptide growth factors can regulate cav-1 expression in NIH 3T3 cells (19). Therefore cav-1 expression and secretion may be stimulated initially by androgens, yet subsequent androgen ablation may select for alternative pathways that sustain cav-1 activities and thus transition the malignant cell into an androgen-insensitive phenotype. As shown in this study, secreted cav-1 can stimulate viability and clonal growth in adjacent prostate cancer cells that do not express cav-1.

The concept of a secreted autocrine/paracrine factor that directly contributes to androgen resistance in prostate cancer is novel and represents an efficient mechanism for maximizing resistance to various proapoptotic stimuli that metastatic cells often encounter during the highly inefficient process of metastasis (20). Our in vivo studies indicating that cav-1-specific antibody delivered i.p. can suppress malignant progression of androgen-insensitive, cav-1-secreting mouse prostate cancer cells are remarkable. These results not only indicate that secreted cav-1 promotes metastasis in vivo, but also raise the possibility of using cav-1 as a therapeutic target for androgen-insensitive disease. It is conceivable that when combined with anti-androgen therapy or potentially with chemotherapy, cav-1-specific antibody therapy may have greater therapeutic activity. Additional studies will be required to address this issue.

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