Caveolin-1 Mediates Testosterone-stimulated Survival/Clonal Growth and Promotes Metastatic Activities in Prostate Cancer Cells

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ABSTRACT

Previously, we demonstrated that up-regulation of caveolin-1 (cav-1) was associated with prostate cancer metastasis, biochemical recurrence after radical prostatectomy, and androgen insensitivity. The objective of this study was to characterize the regulation of cav-1 by testosterone (T) and to test the effects of cav-1 on prostate cancer cell survival/clonal growth and metastatic activities. Our results demonstrated that T up-regulated cav-1 protein levels in part through transcriptional regulation and significantly enhanced survival of prostate cancer cell lines ABAC3 and LNCaP after serum starvation (>40% and >60% increased viability, respectively) and in an extended clonogenic assay (approximately 4-fold and 6-fold increase in colonies, respectively). Importantly, antisense cav-1 inhibited the survival effects of T in these assay systems. Modest but not high levels of adenoviral vector-mediated cav-1 expression alone also significantly increased viability (>40%) and clonal growth (10-fold increase in colonies) after serum starvation. Analysis of spontaneous metastasis in stably transfected antisense cav-1 mouse prostate cancer cell clones demonstrated reduced spontaneous lung node metastasis incidence (13%), spontaneous lymph node metastasis volume (46%), and experimental lung metastasis incidence (40%) compared with vector control cell clones. Surgical castration further reduced spontaneous lymph node metastasis incidence and volume (18% and 28%, respectively) in antisense cancer cell clones, but not in vector control clones. Our studies demonstrate that cav-1 is a downstream effector of T-mediated prostate cancer cell survival/clonal growth and that modest levels of cav-1 can independently promote prostate cancer cell survival/clonal growth and metastatic activities.

INTRODUCTION

Prostate cancer is a continued threat to the lives of tens of thousands of United States men, despite efforts to control this disease through screening of asymptomatic men and the aggressive use of surgery and irradiation therapy for presumed localized disease (1). Unfortunately, many men continue to present with advanced prostate cancer or recur from localized therapy, and there are no curative therapies available for androgen-resistant metastatic disease. Although prostate cancer was shown to be initially responsive to androgen ablation more than 50 years ago (2), there is only minimal understanding at the mechanistic level with regard to the ultimate hormone-resistant state of prostate cancer that is responsible for the exceedingly high mortality rate. Previously, we reported that cav-1 levels were elevated in metastatic mouse and human prostate cancer (3) and that cav-1 positivity had independent prognostic value for cancer recurrence after radical prostatectomy (4). Additional studies demonstrated that suppression of cav-1 levels led to reestablishment of androgen sensitivity in vitro and in vivo and that enforced cav-1 expression could convert androgen-sensitive prostate cancer cells to androgen-insensitive cells (5). Other reports have shown that cav-1 is up-regulated in multidrug-resistant cancer cells, and in some cases, this up-regulation is independent of P-glycoprotein (6–8). More recently cav-1 was shown to suppress c-myc-induced apoptosis in Rat1A and LNCaP cells (9).

cav-1 is the principal component of caveoleae, subinvaginations of the plasma membrane and trans-Golgi network that have been implicated in sphingolipid-cholesterol transport and signal transduction pathways (reviewed in Refs. 10–13). Under some conditions, cav-1 has been shown to suppress growth of specific cell lines in vitro and in vivo (8, 14–17), and it has been suggested that cav-1 functions as a tumor suppressor gene (18). However, specific genetic analysis of cav-1 did not support this contention (19).

Recent studies have indicated that some genes can manifest seemingly opposing functional activities in a context-dependent fashion. One example is the bcl-2 gene that can demonstrate pro- or antiapoptotic activities depending on its level of expression (20). These opposing functions may be related to separate bcl-2 protein domains that have been shown to independently mediate growth arrest or survival depending on cell context (21, 22). Additional examples are the Cox-1 and Cox-2 genes that have been shown to be up-regulated in numerous human malignancies, but overexpression of these genes can suppress growth and induce apoptosis in vitro (23, 24).

Recent studies suggest that the growth-suppressive effects of Cox-1 are not related to its enzymatic activities within the prostaglandin synthesis pathway (24). Overall, these results indicate the need to carefully define the regulation, biological activities, and mechanism(s) of action for these multipotential genes within the context of malignant progression. In this report, we demonstrate that cav-1 is a downstream effector of T-mediated survival activities and that modest but not high levels of cav-1 can promote both cell survival and metastatic activities in mouse and human prostate cancer cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The various metastatic mouse prostate cancer cell lines were generated from tumors initiated by retroviral transduction of the ras and myc oncogenes into fetal prostate tissues from p53 homozygous mutants using the mouse prostate reconstitution model (25). The 148-IPA cell line was established from a primary carcinoma, and 148-1LMd was established from a lung metastasis from the same mouse. The ABAC3 and ABAC5 clonal cell lines were derived from 148-1LMd by introduction of an antisense mouse cav-1 cDNA as described previously (5). Similarly, antisense clone BAC4 was derived from 151-2LMc, a lung metastatic clone from a different mouse (5, 25). ABH11, ABH14, and BHS3 are empty vector clones derived from either 148-1LM or 151-2LMc and used as controls (5). The mouse cell lines were grown in DMEM with 10% FCS. The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection, grown

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3 The abbreviations used are: cav-1, caveolin-1; T, testosterone; SFM, serum-free medium; AR, androgen receptor; MT3, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SFMT, serum-free medium plus testosterone; RSV, Rous sarcoma virus; MOI, multiplicity of infection.

4 C. Ren, L. Garza, Y. Yuan, W. Tian, and T. C. Thompson, unpublished data.

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in RPMI 1640 with 10% FCS, and used at passage 30–60. The appropriate media without serum but with 0.1% BSA were used as SFM. All cells were routinely grown at 37°C with 5% CO2.

**MTT Assay.** Subconfluent cells were trypsinized, collected by centrifugation, and washed once with SFM. A single-cell suspension was then seeded at low cell density (~200 cells/well of a 96-well plate) in SFM alone or with T (Sigma Chemical Co., St. Louis, MO; SFMT). After 3 days, viability of the cells was determined by incubation with 0.05 mg/ml MTT (Sigma Chemical Co., St. Louis, MO; SFMT). After overnight incubation, the medium was replaced with 1 ml of SFM, and adrenoviral vector at different MOIs was added. After 3 h, the medium was removed and replaced with complete medium. After 48 h, the cells were trypsinized for MTT assay and for the preparation of protein lysates. For ATPLite and clonogenic assays, the culture medium was replaced with SFM 48 h after infection, and the cells were subjected to growth/survival factor depletion for 3 days before each assay. Expression of cav-1 was also confirmed in adenoviral vector-infected cells by immunostaining with cav-1 antibody as described previously (4).

A double infection with AdSav-1 was adopted to minimize endogenous cav-1. ABCA3 cells were seeded at 1.0 x 10^5 /well in a 6-well plate and grown overnight. The next day (day 1), cells were infected with the adenoviral-vector at the indicated MOI. A second infection was performed on day 3 (MOI calculations adjusted for increased cell number), followed by another 2-day growth period in complete medium. On day 5, the cells were trypsinized, washed with SFM, and seeded in SFM or SFMT (T = 10 nM) at low cell density as described above for examination of cav-1 protein expression and viability. For the clonogenic assay, SFM- or SFMT-treated cells were detached from a 96-well plate and seeded into 10-cm plates for colony counting as described above.

**In Vivo Metastasis Analyses.** A panel of mouse stable antisense cav-1 clones (ABCAS, ABCB5, and BAC54) and control vector clones (ABH11, ABH14, and BHS3) established from high cav-1-expressing lung metastatic cell lines [148-1LMD or 151-2LMC (3)] were used for orthotopic injection or control vector clones (ABH11, ABH14, and BHS3) established from high cav-1-expressing lung metastatic cell lines [148-1LMD or 151-2LMC (3)] were used for orthotopic injection or tail vein injection into syngeneic 129/SV mice as described previously (26). Each cell clone was injected into eight or nine animals. Orthotopic tumors were established by injection of 5,000 cells, a cell number sufficient to establish a 100% tumor take (26), into the dorsolateral prostate. In some experiments, animals were surgically castrated or received sham surgery 3 days after orthotopic inoculation as described previously (5). Two weeks after orthotopic inoculation, animals were euthanized, the tumor was excised carefully, and the wet weight was recorded. The pelvic and retroperitoneal lymph nodes were excised, placed in formalin, embedded in paraffin, cut into 4–5-μm sections, and stained with H&E for histological examination. The extent of metastasis was assessed qualitatively on the stained slides via computer-assisted image analysis (5). An experimental metastasis assay consisted of the tail vein injection of 50,000 cells. Mice were euthanized after 14 days, and the lungs were weighed and fixed in Bouin’s fixative, and visible lung metastases were counted with the aid of a dissecting microscope at ×10 magnification.

All mice were maintained in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, and all experiments were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals.

**Statistical Analysis.** Statistical analyses were performed with Statview 5.0 (SAS Institute, Inc, Cary, NC). Significance was determined by ANOVA with Fisher’s protected least significant difference.
RESULTS

T Significantly Enhances Cell Survival of Androgen-sensitive Prostate Cancer Cells under Conditions of Growth/Survival Factor Depletion. We initially tested the capacity of T to stimulate viability of both mouse and human prostate cancer cells in vitro using both the MTT assay and the ATPLite assay in SFM with various concentrations of T. As expected, T significantly enhanced cell survival of androgen-sensitive prostate cancer cells after growth/survival factor depletion. In both the MTT assay and the ATPLite assay, maximum viability was observed at 10 nM T for ABAC3 mouse prostate cancer cells with a >40% increase in viable cells compared with SFM (T = 0 nM; p < 0.0001; Fig. 1, A and B). For the human prostate cancer cell line LNCaP, maximum protection was observed at 5 nM T with a >60% (P < 0.0001) increase in viability (Fig. 1, A and B). To analyze the long-term effects of T-stimulated viability, we extended the viability assay to a modified clonogenic assay (see “Materials and Methods”). The results revealed that long-term T stimulation resulted in a ~4-fold increase in colonies for ABAC3 and ~6-fold increase in colonies for LNCaP (Fig. 1C).

Dose-dependent Up-Regulation of cav-1 Protein by T. To analyze the relationship between T levels and cav-1 expression, we determined the dose-dependent effect of T on cav-1 protein expression in mouse and human prostate cancer cell lines. 148-1PA and ABAC3 cells showed maximal induction of cav-1 protein at 20 nM T (Fig. 2). LNCaP cells demonstrated slightly higher sensitivity to T with maximal induction of cav-1 at 5–10 nM T (Fig. 2). These results are in agreement with and extend the findings of a previous report demonstrating increased cav-1 protein levels after T treatment in vitro (27).

Transcriptional Activation of Cav-1 Promoter by T. To determine whether up-regulation of cav-1 by T occurs at the level of transcriptional regulation, we used a luciferase reporter vector under the transcriptional control of the mouse cav-1 promoter, pGL3-mcav-1-luc. The relative activity of the cav-1 promoter was increased more than 2-fold (P < 0.0001) by T in ABAC3 and LNCaP cells (Fig. 3). This activity could be blocked by the addition of 1 μM casodex, a direct AR antagonist (Fig. 3), indicating that the up-regulation of cav-1 by T is mediated by the AR.

Antisense cav-1 Significantly Inhibits the Effects of T on Cell Survival and Clonal Growth. Because T enhanced survival and induced cav-1 expression, we asked whether survival activities in-
Reduced cav-1 Expression in Metastatic Mouse Prostate Cancer Cells Results in Suppression of Metastasis in Vivo. To test the effects of cav-1 expression on metastatic activities in vivo, we analyzed spontaneous (lymph node metastasis from orthotopic tumors) and experimental (tail vein-injected cells) metastasis in a panel of high cav-1-expressing, lung metastasis-derived mouse prostate cancer cell lines; however, when it is expressed at high levels, cav-1 may be toxic to the cells. To determine the long-term effect of cav-1 expression on survival/clonal growth, we also performed a clonogenic assay on infected cells. A significant difference between the effects of AdScav-1 and control vector AdRSV was observed in this extended assay, with ~8–10-fold more colonies for the cav-1 group in a 3-week period (Fig. 5D). To confirm expression of cav-1 in the LNCaP cells, we performed immunohistochemical staining that revealed an absence of cav-1 in uninfected LNCaP cells (Fig. 5E) but readily detectable expression of cav-1 in LNCaP cells infected with AdScav-1 (Fig. 5F).

Cav-1 Expression Promotes Survival and Clonal Growth. To determine whether cav-1 can promote survival in human prostate cancer cells in the absence of T, we infected LNCaP cells with AdScav-1. Dose-dependent expression of human cav-1 was achieved with increasing MOI (Fig. 5A). cav-1 expression increased the relative viability of LNCaP cells after growth/survival factor depletion. Interestingly, the maximum survival protection of cav-1 (>40%; P < 0.0001) was observed at a MOI = 10 (Fig. 5, B and C), which corresponded with a moderate level of cav-1 protein (Fig. 5A). Viability protection was observed with AdScav-1 at MOI 5–50, but not at MOI = 100 or 200 (Fig. 5, B and C). These data clearly suggest that cav-1 can induce survival activities when it is expressed at moderate levels; however, when it is expressed at high levels, cav-1 may be toxic to the cells. To determine the long-term effect of cav-1 expression on survival/clonal growth, we also performed a clonogenic assay on infected cells. A significant difference between the effects of AdScav-1 and control vector AdRSV was observed in this extended assay, with ~8–10-fold more colonies for the cav-1 group in a 3-week period (Fig. 5D). To confirm expression of cav-1 in the LNCaP cells, we performed immunohistochemical staining that revealed an absence of cav-1 in uninfected LNCaP cells (Fig. 5E) but readily detectable expression of cav-1 in LNCaP cells infected with AdScav-1 (Fig. 5F).
lines stably transfected with antisense cav-1 or control vector (5). The growth of the cell lines as orthotopic tumors was compared with that of vector controls after sham surgery (Fig. 6A). The antisense clones were about 10% smaller than the vector clones in the sham-operated animals, but this was not a significant difference (P = 0.226). However, a significant (39%; P < 0.001) decrease in tumor weight was observed in the antisense clones in castrated animals, but not in the vector clones. In these same animals, the extent of spontaneous lymph node metastasis was evaluated in terms of the number of animals with metastases (incidence) and the relative volume of the metastases as determined by computer-assisted microscopic quantitation (Fig. 6B). The antisense clones had less metastatic activity compared with the vector control clones in the sham-operated animals with a 17% decrease in incidence (P = 0.003) and a 52% reduction in relative volume (P < 0.0001). In castrated animals, there was no difference between the vector control clones and the sham-operated animals; however, the antisense clones had a significantly greater decrease in both incidence and volume of lymph node metastasis than the antisense clones in sham-operated animals (18% and 28%, respectively; P < 0.001). To further evaluate metastatic activity, we injected cell clones directly into the tail vein and counted the number of lung metastatic deposits that formed at 2 weeks (Fig. 6C). The antisense cav-1 clones had 40% fewer lung metastases as compared with vector control clones (P < 0.001).

DISCUSSION

Our previous studies have demonstrated that cav-1 is overexpressed in human and mouse metastatic prostate cancer and that overexpression of cav-1 is an independent predictor for recurrence after radical prostatectomy (3, 4). Subsequently, we demonstrated that cav-1 can protect against androgen withdrawal-induced apoptosis in vitro and in vivo and that cav-1 can block c-myc-induced apoptosis in human prostate cancer cells (5, 9). These studies established a foundation on which to more clearly define a role for cav-1 in prostate cancer progression. In this report, we show that T up-regulates cav-1 expression in prostate cancer cells in part through transcriptional regulation. We further demonstrate that increased cell viability and clonal growth in vitro resulting from T treatment is mediated by cav-1 protein and that modest but not high levels of cav-1 alone can independently lead to increased cell viability and clonal growth. Finally, we establish that cav-1 contributes to metastasis in vivo.

In the first series of experiments we demonstrated that T induces cav-1 expression in part at the level of transcriptional regulation. We
demonstrated previously that cav-1 is expressed at very low to non-detectable levels in normal prostate epithelium but is expressed focally in prostate cancer, and further increased expression is associated with prostate cancer metastases (3–5). Together, these results suggest that T is responsible, in part, for inducing cav-1 in prostate cancer cells during progression, but it is not yet clear how the cav-1 gene, which is relatively inactive in normal prostate epithelial cells, becomes responsive to T induction. Conceivably, demethylation could play a role, but previous reports have been inconclusive regarding the role of methylation in cav-1 expression in prostate cancer, and additional studies are needed (19).

To study the effects of T and cav-1 expression on prostate cancer cell survival and clonal growth activities in vitro, we developed a two-step assay system that mimics specific steps of metastasis in vivo. In the first step of this analysis, prostate cancer cells are maintained for 3 days at low density under serum-free conditions, mimicking the reduced growth factor and low density conditions encountered during vascular transit. After this 3-day period, cell viability was analyzed using two independent methods of analysis (the MTT and ATP-Lite assays). Cells were subsequently seeded into a clonogenic assay, which involved a 2–3-week growth period in vitro followed by analysis of colony number. This second step approximates growth at a distal metastatic site and is dependent on continued cell survival. The initial experiments using this assay system demonstrated that T can stimulate cell survival and clonal growth in both mouse and human prostate cancer cells.

Additional experiments in mouse prostate cancer cell lines using adenoviral vector-mediated antisense cav-1 demonstrated that cav-1 induction was responsible, in part, for T-stimulated cell survival/clonal growth in vitro. These results are consistent, in general, with the results of our previous studies that demonstrated that elevated cav-1 levels are associated with androgen insensitivity (5). In the absence of T, it is conceivable that other growth factors stimulate cav-1 expression in prostate cancer. Others have shown that polypeptide growth factors can regulate cav-1 expression in NIH-3T3 cells (29). However, to establish a clear correlation between cav-1 expression and androgen-insensitive human prostate cancer, it will be necessary to demonstrate that cav-1 expression is increased in androgen-insensitive disease and to generate experimental support for androgen-independent regulation of cav-1 expression in androgen-insensitive prostate cancer cells. Additional studies in this area are needed.

The substitution of increased cav-1 expression via infection with AdScav-1 demonstrated that modest levels of cav-1 could also maintain viability in the assay systems described above. The results of the clonogenic assay supported and extended the results of the survival analyses, indicating a severalfold increase in the number of colonies in AdScav-1-infected cells compared with that in control Ad-RSV-infected cells in human (LNCaP) prostate cancer cells.

The data presented in this report, together with our previous studies (9), indicate that relatively modest but not high levels of cav-1 expression can lead to increased cell viability consistent with malignant progression. Overall, these results further reconcile previous reports that have shown that high levels of cav-1 can suppress growth in various cell types (8, 14–17). A recent study indicates that although cav-1 is initially down-regulated in colon cancer cells, reexpression of cav-1 is selected for during the development of drug resistance and metastasis (8). At the molecular level, this dichotomy between the role of cav-1 in tumorigenesis and metastasis may be explained in part by specific interactions between phosphorylated cav-1 and downstream signaling molecules (30). Additional studies are required to define the molecular mechanism(s) through which cav-1 specifically promotes survival/clonal growth in prostate cancer cells.

Finally, we generated in vivo data that support our in vitro studies and demonstrate that experimental reduction of cav-1 expression results in suppression of metastatic activities in vivo. Using stable antisense mouse prostate cancer cell clones, our results indicate that both spontaneous and experimental metastatic activities can be significantly reduced by the suppression of cav-1 expression. The results of our castration studies further suggest that the presence of circulating T together with cav-1 can produce synergistic effects that increase metastatic activities. Interestingly, although a reduction of cav-1 levels suppressed metastatic activities it did not suppress primary tumor growth, demonstrating that the effects of cav-1 in vivo are metastasis specific in this prostate cancer model.

Our results have demonstrated that T can induce cav-1 expression in part through transcriptional regulation and that cav-1 overexpression is, in part, responsible for T-stimulated survival of mouse prostate cancer cells in vitro. Additional studies documented that modest but not high levels of cav-1 can support survival of prostate cancer cells under proapoptotic conditions, i.e., growth/survival factor depletion and low cell density, and promote...
clonal growth in vitro. Finally, analysis of spontaneous and experimental metastasis using stably transfected antisense cav-1 prostate cancer cells confirmed that elevated cav-1 levels contribute to metastasis of prostate cancer cells in vivo. Additional studies will be needed to define the molecular mechanism(s) through which cav-1 contributes to prostate cancer metastasis.

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