Suppression of Prostate Carcinoma Cell Invasion by Expression of Antisense L-Plastin Gene

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Based on the finding that gene expression for the actin-bundling protein L-plastin is inducible by androgen and that L-plastin is overexpressed in malignant epithelium of the prostate, we examined the functional consequences of L-plastin down-regulation in prostate carcinoma cell lines by both transfection and retroviral infection. We constructed retroviral vectors to express two different regions of the L-plastin gene, a 1713-bp 3'-coding portion and a 163-bp 5'-untranslated region, both in antisense orientation. Introduction of either constructs into prostate carcinoma cell lines, PC-3 and its isogenic but metastatic variant PC-3M cells, reduced the growth rates of both cell lines. *In vitro* invasion and motility of PC-3 and PC-3M cells were drastically suppressed (approximately 10-fold) by the expression of the antisense constructs. Evidence was obtained to indicate that L-plastin protein levels were indeed decreased by the antisense expression. The antisense construct for the 5'-untranslated region with the most unique sequence for the L-plastin gene was more effective in down-regulation efficiency compared with the larger antisense construct in the coding region, which maintains homology to other members of the plastin gene family. Cells infected with the 163-bp antisense virus, which were also tested in a nude mouse diaphragm invasion model, showed suppression of *in vivo* invasion of both PC-3 and PC-3M cells. These results suggested that overexpression of L-plastin might be functionally involved in prostate cancer invasion and metastasis, and raised the possibility that L-plastin gene-specific antisense delivery could potentially be a useful approach to interfere with prostate cancer progression *in vivo*. (Am J Pathol 1999, 155:115–122)

The plastins constitute a family of human actin-bundling proteins, isoforms of which display either broad or narrow tissue specificity. The isoform T-plastin is constitutively expressed in epithelial and mesenchymal cells whereas l-plastin expression is restricted to absorptive intestinal

and kidney cells.^{1,2} The isoform L-plastin, which is abundantly expressed in hemopoietic cell lineages, is overexpressed in many types of malignant human solid tumors.^{1,3,4} We observed that, in a steroid-hormone-sensitive prostate cancer model, L-plastin expression was up-regulated by both estrogen and androgen.⁵ It was found that, whereas L-plastin expression is not detectable in normal epithelial cells or epithelium of benign prostatic hyperplasia lesions, L-plastin is overexpressed in carcinoma cell lines and in malignant prostatic epithelium.⁵ The major functional domains of L-plastin (as well as its other two isoforms) are two calcium-binding sites, a calmodulin-binding site, and two tandem actin-binding domains.^{6,7} *In vitro* studies indicate that L-plastin is capable of bundling actin filaments through its actin-binding domains, and this property is negatively regulated by increased calcium concentration through serine residue phosphorylation in the first calcium-binding domain.⁸ Furthermore, there is evidence that overproduction of L-plastin in fibroblastic or epithelial cell systems induces cell rounding and a concomitant reorganization of actin stress filaments into geodesic structures.⁹

Related to the observation of L-plastin overexpression in prostate cancer, another report describes that a new member of the thymosin β family (thymosin β 15) of G-actin binding molecules is up-regulated in highly motile and metastatic Dunning rat prostatic cancer cell lines.¹⁰ Transfection of antisense thymosin β 15 constructs into rat prostate carcinoma cells reduces cell motility. Furthermore, thymosin β 15 levels appear to be elevated in human prostate cancer and correlate positively with the Gleason tumor grade. As cell motility is likely to be linked to coordinated disassembly and reformation of the cortical actin network, it has been proposed that binding of thymosin to actin may enhance the depolymerization phase of this process.¹⁰

We hypothesized that the uniform overexpression of L-plastin in prostate cancer is likely to have a role in prostate cancer progression by directly or indirectly in-

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Figure 1. Schematic representation of pWZLneo-based retroviral vectors. Hatched boxes, M-MLV long terminal repeat (LTR); solid boxes, the internal ribosome entry site (IRES); truncated gag and the neomycin genes as marked. The *EcoRI* cloning site (E) is also shown. The relative positions of ATG and TGA codons in the two constructs are also indicated.

fluencing growth, invasion, and metastasis of the tumors. As a first step in this line of investigation, we report an evaluation of the functional consequence of L-plastin overexpression in prostate cancer cell lines by suppressing the L-plastin expression. The results obtained indicate that *in vitro* characteristics examined, namely, proliferation efficiency and Matrigel invasion, are inhibited by antisense L-plastin sequence expression. Furthermore, we present data to indicate that L-plastin down-regulation manifests a negative influence on *in vivo* invasion of prostate tumor cells in an experimental animal model. Thus, a clinical potential exists that this antisense sequence may suppress progression of human prostate tumors.

Materials and Methods

Cell Culture

Prostate carcinoma cell line PC-3 and its metastatic variant subline PC-3M were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin-penicillin, glutamine, 1X non-essential amino acid solution, and 2X essential amino acid solution (Life Technologies, Grand Island, NY) as described.¹¹ After transfection with antisense L-plastin constructs or infection with an antisense virus, the cells were selected and maintained with 400 μ g/ml G418 in DMEM culture medium.

Construction of Retrovirus Expression Vectors

A 163-bp fragment containing 137 bp of the 5'-untranslated region and 26 bp of the adjacent 5'-translated region of human L-plastin was produced by reverse transcriptase polymerase chain reaction (RT-PCR) with the sense primer C07 (5'-GAATCACTTCCTGCCTTGAC-CAC-3') and the antisense primer C10 (5'-GAATTCT-CATCGGACACTGATCCTC-3'). Both primers were designed to contain an *EcoRI* restriction site at 5' end. The PCR product was then digested with *EcoRI* and cloned into the *EcoRI* site of the pWZLneo retroviral vector.^{12,13} A 1713-bp fragment composed of the 3'-coding region sequence of human L-plastin was also produced by RT-PCR as described⁵ and cloned into TA cloning vector (Invitrogen, Carlsbad, CA). The insert was then cut out of

the vector by *EcoRI* and cloned into the *EcoRI* site of the pWZLneo vector. The antisense constructs for both fragments, the 3'-coding region (AS1) and the 5'-untranslated region (AS2), were confirmed by sequence analysis. A scheme of the pWZLneo retroviral vector and both antisense constructs are shown in Figure 1.

Introduction of Vectors by Transfection

Each of the antisense constructs and the vector-alone plasmid DNA were transfected into PC-3 and PC-3M cells by Lipofectamine (Life Technologies) following the manufacturer's protocol. After 48 hours, the cells were split 1:10 and selected in DMEM containing 600 μ g/ml Genticin for a period of 3 weeks. As the selection of transfected cells was based on the expression of the downstream Neo resistance gene product from the same bicistronic proviral DNA carrying the antisense sequence, the use of the WZLneo vector allowed virtually all selected resistant cells to express the antisense RNA. At least 60 colonies were pooled from each transfection and expanded to generate the AS1, AS2, or vector-only stable cell lines used in the *in vitro* growth and invasion assays.

Isolation and Utilization of Retrovirus Containing the AS2 Sequence

The AS2 or the vector-only DNA was transfected into PA317 amphotropic retrovirus packaging cell line by Lipofectamine, and after 48 hours the cells were split into 1:10 and cultured in DMEM containing 600 μ g/ml Genticin. The surviving clones were trypsinized and expanded individually. The viral titer of the PA317 clones was determined by transfer of the G418 resistance to NIH3T3 cells using serial dilutions of viral supernatant. Clones of cells producing viral titers between 3×10^5 and 5×10^5 infectious units per milliliter were selected for virus collection and for use in the experiments described.

The harvested virus supernatants for AS2 or vector-alone constructs were used to infect PC-3 and PC-3M cells at a multiplicity of infection of approximately 10 to 25 in the presence of 8 μ g/ml polybrene, and the infection procedure was repeated three times for the same culture. The infected cells were selected in the medium contain-

ing 600 $\mu\text{g/ml}$ Geneticin, and individual drug-resistant clones were isolated and expanded for further study. For each construct and each cell type, 15 clones were picked for analysis of L-plastin expression.

Western Blot Analysis

Transfected or infected prostate tumor cells grown in one T75 culture flask were lysed in 250 μl of 2X Laemmli buffer. After boiling for 5 minutes, 10 μl of each lysate was loaded onto a 10% SDS-polyacrylamide gel to resolve proteins. Separated proteins were then transferred to nitrocellulose membranes. The expression of the L-plastin protein was determined using a rabbit anti-L-plastin antibody as previously described.⁵ The membranes were stripped and reprobed with β -actin (Sigma Chemical Co., St. Louis, MO) to assess the protein loading for each lane. The intensities of the bands were determined using NIH Image software. The ratio of the intensity of each L-plastin band from different clones over the intensity of the corresponding β -actin band was calculated and compared for an increase or decrease in L-plastin expression.

Cell Proliferation Assay

Pooled clones of cells transfected with vector-alone or with antisense constructs were seeded at a density of 10^5 cells/60-mm dish in triplicate. The growth rates were monitored over a period of 7 days. Cells were trypsinized every day from the respective plates and counted using a hemocytometer. Each experiment was repeated twice.

Tumor Cell Migration and Invasion Assay

To investigate the effects of decreased expression of L-plastin on the ability of PC-3 and PC-3M cells to migrate through a filter or to invade a biological barrier, we prepared 24-well invasion chambers using Matrigel basement membrane matrix (Becton Dickinson Labware, Bedford, MA) following the manufacturer's suggested procedure. The invasion chamber consists of a 24-well plate with inserts containing an 8- μm pore-size membrane with a thin layer of Matrigel basement membrane matrix, which contains laminin, collagen type IV, heparin sulfate proteoglycan, entactin, and growth factors, including transforming growth factor (TGF)- β and fibroblast growth factor (FGF)-2. Cells were suspended in DMEM containing 0.1% bovine serum albumin and added to the upper chamber at 1×10^5 cells/insert. A conditioned medium obtained by incubating NIH 3T3 cells for 24 hours in serum-free DMEM in the presence of 50 $\mu\text{g/ml}$ ascorbic acid was placed in the lower compartment of invasion chambers as chemoattractants. After 24 hours of culture, the upper surface of the inserts were wiped with cotton swabs, and the inserts were stained with hematoxylin and eosin (H&E). Each experiment was performed twice with each sample in triplicate. The cells that migrated through the Matrigel and the filter pores to the

lower surface were counted under a light microscope with five random high-power fields per insert.

In Vivo Diaphragm Invasion Assay

The diaphragm invasion assay was performed as described previously.¹⁴ Briefly, the cell lines to be tested were grown in culture, trypsinized, washed twice in phosphate-buffered saline, and counted by hemocytometer. Aliquots of 1×10^6 cells were injected intraperitoneally into nu/nu mice, and tumors were allowed to develop for 21 days. In a single experiment, groups of five animals were injected with each of the following cell clones: PC-3 derivatives (vector virus infected V4 and V9 clones and AS2 virus infected 5.4, 8.5, and 10.4 clones) and PC-3M derivatives (vector virus infected VM8 and VM10 clones and AS2 virus infected 11.2, 11.3, and 11.6 clones). At 21 days after inoculation, all animals except two that died before the termination point were sacrificed, the diaphragm from each animal was removed, and the tissue was formalin fixed. Before paraffin embedding, each diaphragm was cut into four sections and arranged in the block such that each paraffin section would contain four different areas of a single diaphragm. Each diaphragm was sectioned three times at different levels of the paraffin block, thus yielding 12 random sections of each diaphragm. The cross sections of the diaphragm were examined for tumor colonies and scored on the basis of basement membrane penetration. A minimum of 12 cross sections from different areas of each diaphragm were examined, and evidence of invasion in any visible tumor resulted in a positive score for invasion. The use of a single event of invasion rather than a percentage of invasive tumors/diaphragm was used to decrease the chances of overestimating the power of the assay. Statistical significance between vector-only and AS2 clones was determined by Fisher's exact test.

Results

Reduction of L-Plastin Expression by Antisense

Levels of expression of L-plastin in antisense transfected or antisense retrovirus infected PC-3 and PC-3M cells were examined by Western blot (Figure 2). Figure 2A shows the results for pooled transfected cells. A higher level of L-plastin expression was seen in the metastatic variant PC-3M cells as compared with the parental PC-3 cells. Although expression of vector alone did not appear to change the levels of L-plastin expression in either PC-3 or PC-3M cells, there was a significantly lower expression in cases where the cells were transfected with the antisense constructs. Although both of the antisense constructs were effective, the 163-bp 5'-untranslated region (AS2) showed a stronger effect on the reduction of L-plastin expression than the 1713-bp coding region (AS1). Figure 2B shows the results from individual AS2 virus infected clones of PC-3 and PC-3M cells. The intensities of the bands recorded on gel pictures were measured and compared by NIH Image software for these clones.

Figure 2. Suppression of L-plastin expression in PC-3 and PC-3M by antisense constructs. **A:** The upper part shows Western blot results of antisense suppression of L-plastin expression in pooled transfected cells for both PC-3 and PC-3M, and the lower part shows Coomassie blue stain of the gels to assess protein loading. Lane 1, parental PC-3; lane 2, parental PC-3M; lane 3, vector-only transfectants of PC-3M; lane 4, AS1 transfected PC-3M; lane 5, AS2 transfected PC-3M; lane 6, parental PC-3; lane 7, vector-only transfected PC-3; lane 8, AS1 transfected PC-3; lane 9, AS2 transfected PC-3. Lanes 1 to 5 and 6 to 9 constitute two different sets of experiments. **B:** The upper part shows Western blot results for antisense L-plastin retrovirus infected clones, and the lower part shows β -actin for the same membranes. Lanes 1 to 5 (PC-3M): lane 1, vector control clone VM4; lane 2, AS2 clone 11.2; lane 3, AS2 clone 11.3; lane 4, AS2 clone 11.6; lane 5, AS2 clone 6.5. Lanes 6 to 10 (PC-3): lane 6, vector control clone V4; lane 7, AS2 clone 8.4; lane 8, AS2 clone 5.4; lane 9, AS2 clone 8.5; lane 10, AS2 clone 10.4.

The expression of L-plastin protein was reduced by approximately 4.5- to 10-fold in antisense L-plastin infected PC-3M and by 3.2- to 10-fold in antisense L-plastin PC-3 cells relative to the corresponding vector-only virus infected clones.

Growth Inhibition of Prostate Carcinoma Cells by Antisense L-Plastin Expression

Antisense L-plastin transfected pools of PC-3 and PC-3M cells were compared for their proliferative abilities with those of vector transfected or untransfected PC-3 and PC-3M cells. At low cell densities, the expression of either antisense L-plastin RNA could reduce the proliferation rates of the transfected cells by approximately 30% to 40% as compared with the vector transfected or untransfected cells as shown in Figure 3. The reduction of the proliferation rate appeared to be slightly greater in antisense transfected PC-3 cells relative to similarly treated PC-3M cells. There was, however, no significant difference in growth rate with respect to AS1 or AS2 expression in either of the cell lines. The down-regulation of L-plastin expression did not induce any obvious morphological changes to PC-3 and PC-3M cells.

In Vitro Migration and Invasion Inhibition of Antisense L-Plastin in Prostate Carcinoma Cells

Invasion chambers were used in this study to investigate the influence of antisense L-plastin expression on the abilities of PC-3 and PC-3M cells to migrate and invade in an *in vitro* system. After 24 hours of culture, cells that invaded through basement membrane Matrigel and mi-

Figure 3. Growth curves of PC-3, PC-3M, and transfectants with vector or antisense L-plastin constructs. **A:** Growth curves for PC-3M, vector, and antisense L-plastin transfected cells. **B:** Growth curves for PC-3, vector, and antisense L-plastin transfected cells.

grated into the other side of the pored filters were checked under a microscope, and the total cell number for each filter was counted. The pooled cells with antisense L-plastin showed drastic reduction of cell number as compared with pooled vector transfected cells (Figure 4). Both AS1 and AS2 constructs produced a significant reduction in invasion and migration properties, although AS2 caused more reduction (87%) than AS1 (68%) in PC-3 cells. The AS2 expression also caused a reduction in invasion (89%) in PC-3M cells relative to AS1 expression (81%). No significant morphological changes in either PC-3 or PC-3M cells were observed. Results of comparative invasiveness for AS2 retrovirus and vector virus infected clones of PC-3 and PC-3M cells are presented in Figure 5. Suppression of invasion for either PC-3 ($P < 0.05$) and PC-3M ($P < 0.01$) was determined to be significant by the Student *t*-test analysis.

Antisense L-Plastin-Mediated Inhibition of *In Vivo* Prostate Tumor Cell Invasion

To determine whether the property of reduced *in vitro* invasion by antisense L-plastin-expressing clones is also maintained *in vivo*, we used a previously described *in vivo* model of invasion in nude mice.¹⁴ Three different clones of each of the PC-3 and PC-3M cell lines infected with AS2 retrovirus were analyzed along with two clones each of vector-only controls. Groups of four animals were in-

Figure 4. Inhibition of motility and invasion by antisense L-plastin expression. Results of invasion assays from pooled PC-3M cells transfected with vector (A), AS1 (B), and AS2 (C) and from pooled PC-3 cells transfected with vector (E), AS1 (F), and AS2 (G) are averaged in columns (D) for PC-3M and columns (H) for PC-3 cells. The bars represent the SD of means of individual experiments.

Figure 5. AS2 expression interferes with invasive ability of individual cell clones. PC-3M clones (A) and PC-3 clones (B) infected with AS2 retrovirus or vector retrovirus were tested using invasion chambers as in Figure 4. Cells that migrated through the membrane and stuck to the lower surface of the filters were counted under the microscope. The bars represent the SD of means of two separate experiments run in triplicate in each case. A: Column 1, vector control clone VM4; column 2, vector control clone VM8; column 3, vector control clone VM10; column 4, AS2 clone 11.2; column 5, AS2 clone 11.3; column 6, AS2 clone 11.6; column 7, AS2 clone 6.5; column 8, AS2 clone 6.2. B: Column 1, vector control clone V4; column 2, vector control clone V9; column 3, vector control clone V11; column 4, AS2 clone 3.4; column 5, AS2 clone 5.4; column 6, AS2 clone 8.5; column 7, AS2 clone 10.4; column 8, AS2 clone 7.2.

jected intraperitoneally with equal amounts (10^6) of cells of each of the clones. After 21 days of incubation, diaphragms were examined for tumor colonies. As indicated in Figure 3, there was a growth difference between vector and AS cell lines, and this difference was maintained *in vivo*. The tumors from vector-only cells were larger than AS2 tumors, but the number of tumor colonies/diaphragm did not appear to be different. The number of diaphragms analyzed in the various groups were as follows: PC-3 vector, 8; PC-3 AS2, 11; PC-3M vector, 7; PC-3M AS2, 13. This analysis revealed that both PC-3 and PC-3M cells were indeed invasive in the assay system used. Figure 6 illustrates this issue (Figure 6, A, B, and E) with vector virus infected PC-3 and PC-3M cells. On infection with AS2 virus the tumors were determined to be less invasive with the majority of diaphragms having no evidence of invasion as illustrated in Figure 6, C–E. Approximately 80% and 70% reduction in invasion was noted in AS2 virus infected PC-3 and PC-3M cells, respectively, compared with the corresponding vector-only controls. These differences were determined to be statistically significant ($P = 0.006$ for PC-3 and $P = 0.005$ for PC-3M).

Discussion

The interest in this study is twofold. First, our results showed that antisense L-plastin gene-mediated down-

regulation of L-plastin expression can inhibit proliferation of prostate carcinoma cells. There is evidence that L-plastin is localized to the focal adhesions and podosomes.⁹ The focal adhesions are indeed the sites of convergence for different signaling pathways involved in the regulation of cell cycle progression, such as the *ras*/MAPK pathway.^{15,16} The observation that disruption of actin filaments can block phosphorylation of MAPK¹⁶ implies a role for actin in the regulation of cellular proliferation. Thus, it seems possible that L-plastin, via its ability to bind actin filaments, may control organization and reorganization of the focal adhesions, and reduced L-plastin expression may interfere with these processes in a way to inhibit the cellular proliferation activity. It is, however, recognized that beyond the levels of L-plastin expression there are many other factors that may contribute to L-plastin's functional activity. For example, although L-plastin is known to bundle actin filaments through its actin-binding domains, this property is negatively regulated by increased calcium concentration through serine residue phosphorylation in the first calcium-binding domain.^{8,17} There is also indirect evidence that serine phosphorylation of L-plastin may have a role in regulating integrin-mediated adhesion in leukocytes.¹⁸ Clearly, much more work will be required to elucidate the mechanisms by which binding interactions of overproduced L-plastin with actin filaments promote cell proliferation.

Second, it is exciting to find that suppression of L-plastin expression in prostate cancer cells can drastically decrease *in vitro* invasion and motility of these cells. These *in vitro* results are strengthened by the observation that antisense-mediated reduction of L-plastin expression in PC-3 or PC-3M results in significant suppression of *in vivo* invasion as assayed by an immunodeficient mouse model of tumor cell invasion. The reduction in diaphragm invasion was approximately fivefold in PC-3 cells and threefold in PC-3M cells. This is an important observation, but the mechanisms by which L-plastin down-regulation inhibits invasion remain unknown. It is reported that cadherin requires anchoring to the actin-based cytoskeleton through catenin to mediate cell-cell adhesion.^{19,20} Furthermore, focal adhesion points are, in fact, adhesive cell membrane structures in which transmembrane receptors such as integrins are clustered to connect the intracellular actin cytoskeleton to the extracellular matrix.^{15,16} Conceivably, because of its binding capability to cytoskeleton structure, L-plastin, when overexpressed, alters the cytoskeleton structure.⁹ This in turn leads to disruption of cell-cell adhesion or cell-extracellular matrix interactions. Conversely, underproduction of L-plastin may restore strong cell-cell and cell-matrix interactions, resulting in reduction of invasive and migratory properties of the affected cells. Although it is speculated that reduction in L-plastin levels affects the actin cytoskeleton and perhaps secondarily adhesion plaques, we do not have any direct evidence in support of those ideas at this time. Obviously, further work will be necessary for elucidating the underlying mechanisms.

Figure 6. Results of *in vivo* invasion assays, illustrated in A to D and summarized in E. Cross sections of diaphragms from mice infected with variously infected tumor cell clones were H&E stained and oriented with the diaphragm above the tumor. Examples shown include vector control PC-3 cell clone V9 (A), vector control PC-3M cell clone VM8 (B), AS2 PC-3 cell clone 8.3 (C), and AS2 PC-3M cell clone 11.6 (D). **Filled arrows** indicated areas of tumor invasion into the smooth muscle of diaphragm. **Open arrows** indicate basement membrane of the diaphragm where no invasion was detected. Data were collected from four animals injected with each clone of PC-3 (V9, V4, AS2 8.5, AS2 5.4, and AS2 10.4) or PC-3M (VM10, VM8, AS2 11.3, AS2 11.2, and AS2 11.6), and results are graphed in E to compare vector only with AS2 in the group of PC-3 and PC-3M cells. Magnification, $\times 50$.

Finally, our previous evidence of uniform overexpression of L-plastin in prostatic malignant epithelium,⁵ combined with the present evidence of inhibition of *in vitro* cell growth, motility, and invasion and *in vivo* tumor cell invasion by reduction of L-plastin expression, presents a case of strong clinical relevance for this line of work. We would like to project that by using vectors with high gene transfer efficiency coupled with strategies of prostate tissue-specific gene expression, it might be possible in the near future to inhibit L-plastin-related motility and invasion properties of the prostate cancer cells, thereby suppressing prostate cancer progression. A perceived

advantage of the approach, in a clinical sense, would be to create a window of opportunity to treat prostate cancer by conventional protocols while tumor cell migration is stalled.

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