Id-1 and Id-2 Proteins as Molecular Markers for Human Prostate Cancer Progression

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ABSTRACT

Purpose: Id proteins are dominant-negative regulators of basic helix-loop-helix transcription factors that control malignant cell behavior in many different tissues. This study aimed to identify the potential role of Id-1 and Id-2 proteins as molecular markers for prostate cancer progression.

Experimental Design: Using the technique of immunohistochemistry, we determined Id-1 and Id-2 expression in a panel of 67 human prostate biopsies. We also manipulated Id-1 and Id-2 expression in LNCaP and PC3 prostate cancer cell lines and determined the effects on invasion in vitro, matrix metalloproteinase secretion, and proliferation.

Results: Both Id-1 and Id-2 proteins were up-regulated during human prostate cancer progression in vivo and were overexpressed in highly aggressive prostate cancer cells. In vitro, constitutive expression of Id-1, and to a lesser extent Id-2, converted nonaggressive LNCaP prostate cancer cells into more proliferative and invasive cells and increased their secretion of matrix metalloproteinases. Conversely, the down-regulation of Id-2 expression in highly metastatic PC3 cells reduced their growth potential and invasiveness.

Conclusions: We propose that both Id-1 and Id-2 proteins control prostate cancer cell phenotypes and could serve as molecular markers of aggressive human prostate cancer.

INTRODUCTION

Prostate cancer is the most common malignancy in men and the second leading cause of male cancer deaths in the United States (1). The majority of prostate cancers are believed to originate from the glands of the peripheral zone of the posterior lobe. The challenge for clinicians is to accurately distinguish the highly invasive, rapidly growing prostate cancers with a propensity for metastasis from those that are minimally invasive, slow growing, and unlikely to metastasize. Traditionally, this distinction is made using a histological grade determined by a pathologist. We hypothesized that Id proteins may represent a new class of reliable molecular markers and could serve as prognostic and/or predictive indicators. Id proteins belong to the helix-loop-helix (HLH) transcriptional regulator family (2). HLH proteins act as obligate dimers, interacting through their HLH domains (3). Id proteins function as dominant antagonists of basic HLH transcription factors by inhibiting their ability to bind E-box DNA sequences within target gene promoters (2, 3). Indeed, Id proteins can specifically dimerize with basic HLH proteins, e.g., ITF-2 or E2A products, but Id–basic HLH heterodimers fail to bind DNA because Id proteins lack the basic domain (2, 3). Although Id proteins are viewed as negative regulators of cell differentiation, they have recently been revealed to participate in cell cycle progression and tumor biology (2, 4).

The focus of our study was on Id-1 and Id-2 proteins. Previous studies in our laboratory have shown that Id proteins control normal and tumoral breast cell phenotypes (5, 6). Moreover, Id-1 and Id-2 expression has been shown to be up-regulated during tumor development and progression in the epidermis (7, 8), colon (9, 10), and pancreas (11, 12). To date, only data regarding Id-1 expression in prostate cancer cells have been reported, and these data are controversial. Two groups described a reduction in Id-1 gene expression in prostate cancer specimens compared with normal prostate (13, 14), whereas another group recently reported the stimulation of prostate cell proliferation by Id-1 (15–18). To our knowledge, nothing is known about the expression and functions of Id-2 in prostate cancer cells in vivo and in vitro or about the roles of Id-1 and Id-2 in prostate cancer cell invasiveness. To investigate the roles of Id-1 and Id-2 genes in prostate cells, we determined by immunohistochemistry (IHC) the expression of both genes in 67 human tumor biopsies from patients with prostate intraepithelial neoplasia or invasive carcinoma. We also manipulated Id-1 and Id-2 expression in LNCaP and PC3 prostate cancer cell lines and determined the effects on invasion in vitro, matrix metalloproteinase (MMP) secretion, and proliferation. On the basis of the data reported here, we propose that both Id-1 and Id-2 act as important molecular switches toward growth, migration, and invasion during human prostate tumor progression and that they could represent promising markers of prognosis and possibly mediators of response to therapy in patients with prostate cancer.

MATERIALS AND METHODS

Constructs and Retrovirus Production. The full-length human Id-1 (1.2 kb) cDNA was cloned in pBabe vector in sense orientation as described previously (5, 6). The coding sequence of the human Id-2 cDNA (a kind gift from Dr E. Hara, Christie Hospital, Manchester, United Kingdom) was excised from...
pcDNA by HindIII/XhoI digestion, blunted, and inserted into pLXSN vector linearized with HpaII to obtain the pLXSN-Id-2 sense construct. The pLXSN-Id-2 antisense plasmid was obtained by BamHI/XhoI digestion of pcDNA-Id-2 and pLXSN, followed by ligation. Each retroviral construct was transfected into the TSA54 packaging cell line (Cell Genesis, Foster City, CA) by use of calcium phosphate. Twenty-four h after transfection, the TSA54 culture medium containing the packaged retroviral vectors was harvested and frozen at −80°C.

Cell Culture and Retrovirus Infection. The human prostate cancer cell lines PC3 and LNCaP were purchased from the American Tissue Culture Collection. Prostate cells were maintained in RPMI 1640 (University of California, San Francisco, CA) supplemented with 10% fetal bovine serum for growing conditions or with 5 μg/ml insulin (Sigma) for serum-deprived experiments. PC3 and LNCaP cells were stably infected with the previously described constructs. Cells were selected in the appropriate selective medium supplemented with neomycin (900 μg/ml) or puromycin (1.2 μg/ml) for a period of 8–14 days. The resistant colonies were pooled.

RNA Isolation and Northern Analysis. Total cellular RNA was isolated and purified as described previously (6). Samples (20 μg of total RNA) were fractionated by electrophoresis through denaturing formaldehyde-agarose gels and transferred to nylon membranes (Hybond-N; Amersham Corporation). The blots were hybridized with 32P-labeled probes prepared by random oligonucleotide priming and exposed for autoradiography.

Western Analyses. Cells were scraped and lysed, and the protein extracts were separated by SDS-PAGE using 10% acrylamide gels and blotted on nitrocellulose membranes. After blocking, blots were hybridized with rabbit anti-Id-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Id-2 (Santa Cruz Biotechnology) as well as anti-actin (Chemicon) diluted in Tris-HCl-buffered saline (TBS; pH 7.6) containing 0.1% Tween 20 and 10% nonfat dry milk. Membranes were incubated with horseradish peroxidase-labeled secondary antibodies, and the signal was detected by ECL chemiluminescence.

Zymography. Proliferating cells were cultured in serum-free medium for 2 days, at which time the conditioned medium was collected and concentrated 20–30-fold by use of 5-kDa cutoff filters (Vivaspin 6; Vivascience). The concentrated medium was analyzed on gelatin substrate gels as described previously (5). Gelatinase activities were visible as clear bands indicative of proteolysis of the substrate protein.

Boyden Chamber Invasion Assay. Assays were performed as described previously (6) in modified Boyden chambers with 8 μm pore filter inserts for 24-well plates (Collaborative Research). Filters were coated with 12 μl of ice-cold Matrigel (11 mg/ml protein; Collaborative Research) either undiluted for PC3 cells or diluted to 10% with plain RPMI 1640 for LNCaP cells. Cells (1 × 105/well) in 200 μl of serum-free RPMI 1640 supplemented with 5 μg/ml insulin (Sigma) were added to the upper chamber. After 16–24 h of incubation, cells were fixed with 2.5% glutaraldehyde in PBS. Cells were assayed in quadruplicate or hexaplicate, and at least two independent experiments were carried out.

[3H]Thymidine Labeling and Cell Growth. Cells cultured with 0.5% fetal bovine serum on coverslips in 30-mm dishes were given [3H]methylthymidine (10 μCi/ml; 60–80 Ci/mmol; Amersham) for the last 16 h of the experiment. After rinsing, cells were fixed, stained with 4’,6-diamidino-2-phenylindole, coated with NTB2 emulsion (1:2 dilution; Kodak), exposed for 24 h, developed with D-19, fixed in Rapid-Fix (Kodak), and mounted on a glass slide. The percentage of labeled nuclei was calculated. Each experiment was carried out in triplicate, and at least two independent experiments were performed.

IHC. Prostate biopsies containing cancer cells were obtained from 67 patients at California Pacific Medical Center. A total of 57 patients had invasive carcinomas, and 10 had prostatic intraepithelial neoplasia (PIN). Gleason scores of the 57 invasive cancers were as follows: grade 5/5 (n = 2), 5/4 (n = 10), 4/4 (n = 12), 4/3 (n = 9), 3/3 (n = 9), 3/2 (n = 11), and 2/2 (n = 4). The 10 PINs were not graded. Slides were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. Slides were treated as described previously (6) and incubated overnight at 4°C with 1.5 μg/ml anti Id-1 antibody or 1.2 μg/ml anti Id-2 antibody (same antibodies as for the Western analyses) in TBS containing 1% BSA. The slides were washed in TBS, incubated with biotinylated swine antirabbit F(ab)’2 (1:400) for 30 min, and then incubated with 1:500 streptavidin-horseradish peroxidase for 30 min. After the slides were washed in TBS, peroxidase was visualized by incubation in 0.5 mg/ml diaminobenzidine-H Cl and 0.03% hydrogen peroxide in TBS. Normal rabbit IgG and Id-1 or Id-2 blocking peptides were used as control. All of the sections were briefly counterstained with Mayer’s hematoxylin solution, rinsed, dehydrated in graded alcohols, transferred in xylene, and mounted.

The prostate biopsies containing invasive adenocarcinoma were scored and graded according to the Gleason system (19, 20). The PINs were not graded. All foci of invasive adenocarcinoma in tissue sections judged to have technically acceptable immunostaining were assessed for the percentage of cells staining positively for Id-1 and Id-2 in the nucleus, cytoplasm, or both and for the predominant staining intensity (0–4+). The percentage of cells staining positive was recorded on an ordered categorical scale (e.g., 0 = 0–10%; 1 = 11–20%; 2 = 21–30%; 3 = 31–40% for primary and secondary pattern-scored areas. The cut point for positive staining was defined as >10% of tumor cells with a staining intensity >1+.

RESULTS

Id-1 and Id-2 Expression in Human Prostate Biopsies. IHC for Id-1 and Id-2 was performed on 67 fine-needle prostate biopsies from human patients. In the areas of normal prostate epithelial cells, Id-1 and Id-2 expression was undetectable, whereas strong signals were detected in the most poorly differentiated cancerous glandular epithelia (Fig. 1A). Although Id-1 and Id-2 proteins were predominantly detectable in the cytoplasm of epithelial cancerous cells, strong nuclear staining was also observed for Id-2 in the higher prostate tumor grades. Id-2, and to a lesser extent Id-1 protein, was also expressed at moderate levels within PINs (Fig. 1A, panels b1–b3). High-grade PINs are thought to represent the precursors of invasive prostate carcinomas (21, 22). The analysis of these IHC data, based on the intensity of Id immunostaining, is summarized in Fig. 1B. The correlation of Id-1 and Id-2 protein expression with Gleason
pattern scores is presented in Table 1. The correlation between pattern score (coded 0–5) and mean Id-1 stain intensity was 0.97, and the correlation between pattern score and median Id-1 stain intensity was 0.95. For Id-2, the correlation between pattern score and mean stain was 0.97, and the correlation between pattern score and median stain was 0.98. All correlations were statistically significant ($P < 0.001$).

We conclude that Id-1 and Id-2 immunostaining is positively correlated with prostate cancer progression and that Id-1 and Id-2 proteins are overexpressed in highly aggressive prostate cancer cells (as measured by grade).

**Id-1 and Id-2 Expression in Human Prostate Cancer Cell Lines.** To further assess the roles of Id-1 and Id-2 proteins, we investigated their expression in prostate cancer cells in culture. As a model for nonaggressive prostate cancer cells (corresponding to low-grade carcinomas), we chose LNCaP cells, which are androgen- and estrogen-receptor-positive cells (23). As a model for highly aggressive and metastatic cells (corresponding high-grade carcinomas), we chose PC3 cells, which were originally isolated from a bone metastasis and are highly invasive and tumorigenic in athymic nude mice (23). We found that high amounts of Id-2 were expressed in the aggressive PC3 cells, contrasting with the weak signal observed in the less-invasive LNCaP cells (Fig. 2). Similarly, Id-1 was easily detectable in PC3 cells, whereas it was undetectable in LNCaP cells (Fig. 2). These *in vivo* and *in vitro* observations (Figs. 1 and 2) therefore suggested that both Id-1 and Id-2 might be up-regulated, and potentially required, during prostate cancer cell progression. As a next step, we inves-

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**Fig. 1.** A, immunohistochemical detection of Id-1 and Id-2 proteins in human prostate biopsies. Tissue sections from fine-needle prostate biopsies were immunostained for Id-1 (panels a2, b2, c2, d2, e2, and f2) and Id-2 (panels a3, b3, c3, d3, e3, and f3) proteins. Panels a1–e3, normal prostate glands; panels b1–b3, prostate intraepithelial neoplasia; panels e1–e3, prostate cancer, Gleason pattern score 2 (G-2); panels d1–d3, prostate cancer, Gleason pattern score 3 (G-3); panels e1–e3, prostate cancer, Gleason pattern score 4 (G-4); panels f1–f3, prostate cancer, Gleason pattern score 5 (G-5). Nuclei are visible in blue (hematoxylin staining in a1, b1, c1, d1, e1, and f1). Pictures of prostate epithelial glands are ×400 magnification. Controls presented in panels f1–f3 (smaller frames) use either Id-1-blocking peptide (f2) or Id-2-blocking peptide (f3). B (left), data analysis of the *in vivo* immunodetection of Id-1 and Id-2 protein within prostate glandular epithelia. The intensity of the immunohistochemical (IHC) detection was graded from 0 to 4, and the percentage of cells per gland stained for each intensity level is shown as follows: □, grade 0; □, grade 1; □, grade 2; □, grade 3; ■, grade 4+. (Right), the data are presented as a statistical analysis. Prostate gland phenotypes are indicated under the graphics as Normal, PIN (prostate intraepithelial neoplasia); and G-2 to G-5 (prostate cancer graded 2–5 with the Gleason pattern score).
tigated the effects of modifying Id-1 and Id-2 protein levels in LNCaP and PC3 cells.

Modulation of Id-1 and Id-2 Expression in Nonaggressive LNCaP Cells. Because Id-1 mRNA was undetectable and Id-2 mRNA was low in the nonaggressive LNCaP cells, contrasting with the highly metastatic PC3 cell line (Fig. 2), we overexpressed either Id-1 or Id-2 in LNCaP cells. Western blotting confirmed the exogenous expression of Id-1 and Id-2 proteins (Fig. 3A). LNCaP Id-1S cells expressed very high levels of Id-1, and LNCaP Id-2S cells expressed significantly higher amounts of Id-2 compared with their respective controls.

Constitutive Id-1 and, to a Lesser Extent, Id-2 Expression Increases LNCaP Cell Invasiveness in Vitro and MMP Secretion. A critical step toward malignancy lies in the spreading of cancer cells from their originating tissue to other body areas, such as bone for prostate cancer. LNCaP Id-1S and Id-2S cells, as well as the control cells, were assayed with the in vitro invasion Boyden chamber assay. LNCaP cells overexpressing Id-1 protein were 4-fold more invasive than the LNCaP control cells. LNCaP Id-2S cells were ~1.5-fold more invasive than the control cells (Fig. 3B). Thus, the constitutive expression of Id-1, and to a lesser extent Id-2 protein, significantly induced an invasive and migratory phenotype in the nonaggressive LNCaP cells.

The significant increase in the ability of LNCaP Id-1S cells to invade and migrate through the extracellular matrix in vitro suggested that Id-1 protein may induce the expression of extracellular matrix-degrading proteases. Indeed, the overexpression of Id-1 in LNCaP Id-1S cells induced a significant increase in the secretion of the active form of MMP-2 (62 kDa; Fig. 3C). In the LNCaP Id-2S population, Id-2 up-regulation was associated with only a slight increase in invasiveness, and therefore no significant difference in the pattern of MMP expression was detected (data not shown).

Constitutive Expression of Id-1 and Id-2 Stimulates LNCaP Cell Proliferation. We next determined the in vitro growth capacity of the transduced cell lines by the technique of [3H]thymidine nuclear incorporation. In low-serum conditions, the rate of proliferation of LNCaP Id-1S cells was increased 2-fold compared with the LNCaP control cells, and the proliferative capacity of LNCaP Id-2S was at least 1.5-fold higher than the control cells (Fig. 3D). Thus, both Id-1 and Id-2 proteins were able to confer a growth advantage to LNCaP cells in low-serum conditions.

We conclude that constitutive Id-1 or Id-2 expression is sufficient to confer a more aggressive phenotype to LNCaP cells. Id-1, and to a lesser extent Id-2, triggered (a) cellular invasiveness and migration, possibly through MMP-2 secretion for Id-1, and (b) an increase in the rate of proliferation.

Modulation of Id-1 and Id-2 Expression in Highly Aggressive PC3 Cells. PC3 cells showed high levels of endogenous Id-2 and presented a detectable signal for Id-1 (Fig. 2). We therefore determined the effects of down-regulation of Id-2 as well as the effects of an increase in Id-1 expression on the phenotype of PC3 cells. To reduce Id-2 expression, we used antisense technology and confirmed that PC3 Id-2AS cells expressed significantly less Id-2 protein (Fig. 4A). We also determined whether an increase in Id-1 expression in PC3 cells could increase their aggressiveness. As expected, Western blotting confirmed the up-regulation in Id-1 expression in the PC3 Id-1S cell population (Fig. 4A).

Table 1

<table>
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<th>Gleason score</th>
<th>Id-1 Count</th>
<th>Id-1 Mean</th>
<th>Id-1 Median</th>
<th>Id-1 SE</th>
<th>Id-2 Count</th>
<th>Id-2 Mean</th>
<th>Id-2 Median</th>
<th>Id-2 SE</th>
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<td>0.08</td>
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<td>1.10</td>
<td>0.17</td>
<td>30</td>
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<td>2.00</td>
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<td>0.28</td>
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<td>4.00</td>
<td>0.24</td>
<td>13</td>
<td>3.82</td>
<td>4.00</td>
<td>0.09</td>
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* Counts, number of slides containing this type of grade.

** Mean and median, average and median intensity across all slides containing this type of grade.

N/B, normal/benign; PIN, prostate intraepithelial neoplasia.

Fig. 2 Northern blot analysis of Id-1 and Id-2 RNA levels extracted from LNCaP and PC3 prostate cancer cell lines. Id-1 and Id-2 mRNA are indicated (arrows). The 28S and 18S rRNA bands are shown as controls for RNA integrity and quantitation.
Both Id-1 and Id-2 Proteins Are Positive Regulators of PC3 Invasiveness in Vitro. PC3 Id-2AS cell invasiveness was significantly reduced, by 75–80%, compared with PC3 control cells (Fig. 4B). Thus, a significant decrease in Id-2 expression could convert PC3 cells into less invasive and migratory cells. However, PC3 Id-2AS cells did not show significant variations in gelatinases as well as caseinases compared with the controls (data not shown). Id-2 may therefore exert its function through the up-regulation of proteases not detectable by gelatinase or caseinase gels. Conversely, the constitutive up-regulation of Id-1 protein in PC3 cells correlated with a slight increase in their invasive phenotype.

Both Id-1 and Id-2 Are Involved in Regulation of PC3 Cell Proliferation. Significant effects of Id-1S and Id-2AS on cell proliferation were observed when PC3 cells were cultured in low-serum conditions (Fig. 4C). The increase in Id-1 expression in PC3 Id-1S cells was associated with a concomitant 1.75-fold increase in the rate of proliferation. Conversely, the down-regulation of Id-2 reduced the proliferative ability of PC3 cells by 2-fold. These results indicate that both Id-1 and Id-2 are able to confer a growth advantage to PC3 prostate cancer cells when cells are maintained in low serum.

These experiments on PC3 cells therefore demonstrated that a reduction in Id-2 protein expression triggered a decrease in the aggressive and invasive phenotype. Conversely, Id-1 protein overexpression further increased PC3 cell aggressiveness.

DISCUSSION

In recent years, progress has been made in elucidating the mechanisms that regulate the transformation of prostate epithelial cells. Although some major molecular pathways have been suggested to be dysregulated, implicating PTEN, MXI1, insulin-
Id-1 and Id-2, and actin proteins expressed in transduced PC3 cells. Lanes 1–4 correspond to PC3 CtlpBabe, PC3 Id-1S, PC3 CtlpLXSN, and PC3 Id-2AS cells, respectively. As indicated, the top two bands in Lanes 1 and 2 show Id-1 immunodetection, whereas the top two bands in Lanes 3 and 4 show Id-2 immunodetection. The actin band is shown as a control. B, Boyden chamber invasion assay comparing the invasive ability of the different PC3 cell populations. Column 1, control cells; column 2, PC3 Id-1S cells; column 3, PC3 Id-2AS cells. Data are presented as percentage of control (bars, SD). Within each chamber, a 100% Matrigel layer was coated on the pore filter, and 1 × 10^5 cells were added. C, [3H]thymidine incorporation in the different PC3 cell populations. Column 1, control cells; column 2, PC3 Id-1S cells; column 3, PC3 Id-2AS cells. Data are presented as percentage of control (bars, SD).

Fig. 4  Modulation of Id-1 and Id-2 expression in PC3 cells. A, Western blot of Id-1, Id-2, and actin proteins expressed in transduced PC3 cells. Lanes 1–4 correspond to PC3 CtlpBabe, PC3 Id-1S, PC3 CtlpLXSN, and PC3 Id-2AS cells, respectively. As indicated, the top two bands in Lanes 1 and 2 show Id-1 immunodetection, whereas the top two bands in Lanes 3 and 4 show Id-2 immunodetection. The actin band is shown as a control. B, Boyden chamber invasion assay comparing the invasive ability of the different PC3 cell populations. Column 1, control cells; column 2, PC3 Id-1S cells; column 3, PC3 Id-2AS cells. Data are presented as percentage of control (bars, SD). Within each chamber, a 100% Matrigel layer was coated on the pore filter, and 1 × 10^5 cells were added. C, [3H]thymidine incorporation in the different PC3 cell populations. Column 1, control cells; column 2, PC3 Id-1S cells; column 3, PC3 Id-2AS cells. Data are presented as percentage of control (bars, SD).

like growth factor 1, or E-cadherin (22), the molecular mechanisms underlying the development and progression of prostate cancer are still unclear. In this study, we report that Id-1 and Id-2 proteins, two regulators of the basic HLH transcription factor network, are able to control aspects of prostate cancer cell phenotype. We established that up-regulation of both Id-1 and Id-2 correlated with the level of aggressiveness of human prostate cancer in vivo (as measured in tumors by grade) and in vitro.

By IHC, very high levels of Id-1 and Id-2 proteins were detected in the most poorly differentiated areas of human prostate carcinoma. As described previously for other types of tissue (6), Id-1-positive cells showed prominent cytoplasmic staining. This was expected because Id-1 protein lacks nuclear localization signals. However, Id-2, in addition to its cytoplasmic localization, was also detected in the nuclei of cells. We recently determined that Id-2 protein is expressed predominantly in the nuclei of mammary epithelial cells (24). Moreover, in cultured astrocytes, Id-2, but not Id-1, is translocated from the cytoplasm to the nucleus in response to serum (25). The physiological relevance of the subcellular localization of Id proteins has yet to be established (2).

The up-regulation of Id proteins occurs mainly in advanced prostate cancers, as shown previously for Id-1 in other types of carcinomas, such as breast (5, 6, 26), cervical (27), and ovarian (28) cancers. The co-up-regulation of Id-1 and Id-2 has also been observed previously in the unregulated proliferation and invasiveness of, and correlated with disease progression and propensity to metastasize in, other epithelial tissue-derived cancers, including pancreatic carcinoma (11, 12), colorectal cancer (9, 10), and epidermal keratinocyte tumors (7, 8). The in vivo up-regulation of Id-1 protein during prostate cancer progression that we detected is in agreement with a previous IHC analysis (18), although not with previous microarray analysis suggesting lower levels of expression of Id-1 mRNA in prostate cancer areas compared with normal prostate tissues (13, 14). Consistent with our in vivo results, Id-1 and Id-2 proteins were specifically up-regulated in the aggressive PC3 prostate cancer cells, contrasting with the nonaggressive LNCaP cells. Taken together, these data suggest a potential correlation between dysregulation of Id gene expression and prostate cancer progression.

Even if 25–50% of PINs, as well as pattern score 2 carcinomas, expressed little or no Id-1 and Id-2, Id proteins may be implicated in prostate cancer development. A large proportion of PINs exhibited relatively high expression of Id-2 protein, and to a lesser extent Id-1 protein. Because PINs are thought to represent the precursors of early invasive prostate carcinomas (21, 22), we suggest that Id proteins might be potential useful markers for early detection of aggressive prostate cancer.

To specifically determine the contributions of Id-1 and Id-2 proteins in the regulation of prostate cancer cell phenotypes, we modulated their expression in vitro, using LNCaP and PC3 cell lines. We found that constitutively high expression of Id-1 or Id-2 converted LNCaP and PC3 cells into more aggressive cells, with higher cell growth capabilities and invasive capacities. Both Id-1 and Id-2 also suppressed the apoptotic index of these two cell lines, which, however, originally presented low levels of apoptosis (data not shown). Conversely, the down-regulation of Id-2 in PC3 cells was associated with a less aggressive phenotype, as demonstrated by a lower rate of invasion, migration, and proliferation (see Fig. 4) as well as an increase in the apoptotic index (data not shown). One of the molecular mechanisms by which Id-1 and Id-2 may exert their regulation of invasiveness is the control of MMP secretion and activation. Indeed, we found that the up-regulation of Id-1 protein in LNCaP cells could significantly increase the secretion of one of the matrix metalloproteinases, MMP-2. MMPs were previously shown to play a significant role in prostate tumor development from invasion and metastasis to endothelial cell migration and neo-angiogenesis (29). Moreover, the regulation of secretion of a 120-kDa gelatinase (5) and MMP-2 (30) by Id proteins has already been suggested in mammary epithelial cells and endothelial cells, respectively.

The fact that we determined that Id-1 and Id-2 are positive regulators of prostate cancer cell growth is in agreement with previous reports. For example, the inactivation of the p16/pRB pathway was related to Id-1 overexpression in LNCaP cells (17), and up-regulation of Id-1 could consequently lead to LNCaP cell proliferation. Id-2 protein is also a crucial regulator of the cell cycle machinery (2, 4). Indeed, high levels of Id-2 protein have the ability to functionally inhibit the Rb tumor suppressor pathway, a process that is widely viewed as a hallmark of cancer and leading to cell cycle activation. Id-2 protein overexpression...
in prostate cancer cells could consequently control their growth potential.

Finally, the cellular and molecular evidences presented here contribute to an understanding of prostate cancer progression. We believe that one of the major determinants could be the uncontrolled up-regulation of Id-1 and Id-2. These two genes would play a crucial role during the pathophysiological progression of nonaggressive prostate cancers to invasive and metastatic phenotypes.

In conclusion, our in vitro and in vivo data suggest that both Id-1 and Id-2 proteins represent promising prognostic and therapeutic predictive markers of human prostate cancer. We are presently investigating the relationship between their expression and patient outcome. Moreover, and as described previously for breast cancer therapy (31), we suggest that targeting Id expression by use of antisense constructs would not only reduce the invasion of prostate cancer cells in vitro but also their ability to metastasize in vivo.

ACKNOWLEDGMENTS

We thank Dr. Andrew P. Smith for editing, Dr. Sylvia Fong for critical reading of this manuscript, and Drs. Nancy M. Lee, Jarmail Singh, and Tomoki Sumida for helpful scientific discussions.

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